The University Of Sheffield.

Access To Thesis.



- A bound copy of every thesis which is accepted as worthy for a higher degree, must be deposited in the University of Sheffield Library, where it will be made available for borrowing or consultation in accordance with University Regulations.
- All students registering from 2008–09 onwards are also required to submit an electronic copy of their final, approved thesis. Students who registered prior to 2008–09 may also submit electronically, but this is not required.

Author: LAUREN MICHELLE JENNY BUCK Dept: BMS

Thesis Title: THE ZEBRAFISH AS AN IN VIVO MODEL OF DRUG-INDUCED AUDITORY AND VESTIBULAR IMPAIRMENT Registration No: 080124201

**For completion by all students:**

Submit in print form only (for deposit in the University Library): ☐

Submit in print form and also upload to the *White Rose eTheses Online* server: In full ☑

Edited eThesis ☐

**Please indicate if there are any embargo restrictions on this thesis. Please note that if no boxes are ticked, you will have consented to your thesis being made available without any restrictions.**

| Embargo details: (complete only if requesting an embargo to either your print and/or eThesis) | Embargo required? | | Length of embargo (in years) |
|---|---|---|---|
| Print Thesis | Yes ☐ | No ☑ | |
| eThesis | Yes ☑ | No ☐ | 5 |

**Supervisor:** I, the supervisor, agree to the named thesis being made available under the conditions specified above.

Name: DR. TANYA WHITFIELD Dept: BMS

Signed: TWhitfield Date: 12/11/12

**Student:** I, the author, agree to the named thesis being made available under the conditions specified above.

I give permission to the University of Sheffield to reproduce the print thesis in whole or in part in order to supply single copies for the purpose of research or private study for a non-commercial purpose.

I confirm that this thesis is my own work, and where materials owned by a third party have been used copyright clearance has been obtained. I am aware of the University's *Guidance on the Use of Unfair Means* (www.sheffield.ac.uk/lets/design/unfair)

I confirm that all copies of the thesis submitted to the University (including electronic copies on CD/DVD) are identical in content.

Name: LAUREN MICHELLE JENNY BUCK Dept: BMS

Signed: Date: 12/11/12

**For completion by students also submitting an electronic thesis (eThesis):**

I, the author, agree that the University of Sheffield's eThesis repository (currently WREO) will make my eThesis available over the internet via an entirely non-exclusive agreement and that, without changing content, WREO may convert my thesis to any medium or format for the purpose of future preservation and accessibility.

I, the author, agree that the metadata relating to the eThesis will normally appear on both the University's eThesis server and the British Library's EThOS service, even if the thesis is subject to an embargo. I agree that a copy of the eThesis may be supplied to the British Library.

I confirm that the upload is identical to the final, examined and awarded version of the thesis as submitted in print to the University for deposit in the Library (unless edited as indicated above).

Name: LAUREN MICHELLE JENNY BUCK Dept: BMS

Signed: Date: 12/11/12

**THIS SHEET MUST BE BOUND IN THE FRONT OF THE PRINTED THESIS BEFORE IT IS SUBMITTED**

# The Zebrafish as an In Vivo Model of Drug-Induced Auditory and Vestibular Impairment

Thesis submitted to the University of Sheffield
for the degree of Doctor of Philosophy

by

**Lauren Michelle Jenny Buck**

Department of Biomedical Science

November 2012

## Disclaimer

Some of the work described in this thesis has already appeared in published form (Buck et al., *Hearing Research,* 2012). I was the primary author on this paper and was responsible for writing the text. Some sections of this thesis (for example in the Materials and Methods chapter) were reproduced in the *Hearing Research* paper My supervisor and I can confirm that both the text in the paper and the text in the thesis is all my own work.

Lauren Buck 

Dr Tanya Whitfield 

# Acknowledgements

Firstly, I would like to extend a special thank you to my supervisors Dr. Tanya Whitfield, Dr. Matthew Winter and Dr. Will Redfern for their excellent support over the last four years. Specifically, I would like to thank Tanya for her excellent guidance, her compassion and understanding, and above all, the excellent cake baking. I'd like to thank Matt for helping me to settle in to life in Devon, for his direction and for sharing his office with me for six months. Finally, I'd like to thank Will for his encouragement, advice and generosity (especially for the wonderful Austrian hat). Additionally, I'd like to thank my advisors Prof. Matthew Holley and Dr. Freek van Eeden for their interest in the project and their time.

I am indebted to Alan Sharpe and Nick Monk for their help with statistical analysis, and to the BEL engineering department, Pete Nicholson and Robert Chandler for superb technical assistance. I would have been completely lost without the help of the Viewpoint team, who provided much equipment and expertise (merci, Yann and Fred). Thank you to aquarium staff at both the MRC CDBG Sheffield and at BEL aquaria for taking good care of the zebrafish.

On a more personal note, thank you to all past and previous members of the Whitfield laboratory for all of the emotional support and coffee, and a number of friends on D floor for fun times. Dziękuję bardzo, Aga for being my write-up room partner in crime! Thank you to friends and colleagues at BEL for making my time in Devon so enjoyable. I am grateful to Tina and Kelly for being wonderful housemates, to Tom, Amelia and Sarah for the early morning gym sessions that kept me sane, to Alex for not eating my slice of banana cake (!) and to Malcolm for the "friendly" rugby rivalry.

Most of all, I am hugely thankful for such wonderful family (Dave, Tina, Lou, Tom and Leonie) and brilliant friends. The people closest to me have had to cope with many a rant and a few tears along the way, and I can never say thank you enough. Your encouragement, wise words and unconditional support have carried me through.

# Abstract

A variety of marketed drugs are known to cause damage to the hearing and/or balance systems in humans (ototoxicity). The absence of standard testing protocols to identify ototoxic effects at the pre-clinical stage is one major reason for the incidence of these adverse effects in the clinic.

The zebrafish (*Danio rerio*) has been proposed as a suitable model organism to determine the ototoxicity of compounds early on. Free-swimming zebrafish larvae possess rudimentary hearing and balance systems that consist of inner ear and lateral line structures, with specialised hair cells similar to those of the human inner ear.

The studies described in this thesis examine the pathological and functional consequences of hair cell loss in the lateral line and otic vesicle of larval zebrafish, following exposure to a range of reported human ototoxins. The aim of these studies was to assess the validity and translational capability of the larval zebrafish as a microplate-scale in vivo model of mammalian inner ear hair cell responses to ototoxin exposure.

Histological analyses of hair cell damage were performed using the vital dyes DASPEI and FM1-43FX, in addition to *Tg(pou4f3::mGFP)s356t* and *i193* transgenic lines, which mark hair cells. Results of the histological investigations showed hair cell damage to occur in a concentration-dependent manner after exposure to representatives from a range of drug classes. These classes include the aminoglycoside antibiotics, salicylates and platinum-based chemotherapeutics. Injection of ototoxins into the ear was also sufficient to induce hair cell damage to specific sensory maculae.

The functional impact of hair cell damage was investigated using startle, rheotaxis and seeker response assays, which harnessed the natural behavioural responses of the larvae. A novel semi-automated startle assay was developed and optimised to study the effects of hair cell damage on the acoustically-evoked high-speed escape response. Functional assessment revealed the significant attenuation of startle, rheotaxis and underwater motion detection following exposure to compounds that had previously induced hair cell damage.

Additional investigations into the mechanisms underlying hair cell damage for one ototoxin, cisplatin, are also described. Chemical manipulation of one component of the proposed mammalian pathway of damage was able to significantly protect hair cells in the zebrafish, suggesting conservation of damage mechanisms between zebrafish and higher vertebrates. Collectively, the data described in this thesis provide evidence to support the use of zebrafish as an in vivo model of ototoxicity.

# List of Abbreviations

| | |
|---|---|
| ABR | Auditory brainstem response |
| ADME | Absorption, distribution, metabolism and excretion |
| ADR | Adverse drug reaction |
| AER | Auditory-evoked (high speed) responses |
| AHL | Action on Hearing Loss |
| ALD | Average large distance |
| ALL | Anterior lateral line |
| ANOVA | analysis of variance |
| BSA | Body surface area |
| cDNA | Complementary DNA |
| ChIn | Chemically induced inflammation |
| CNT | Cognition network technology |
| CPP | Conditioned place preference |
| Ctr1 | Copper transporter 1 |
| dB SPL | Decibels sound pressure level |
| dB TP | Decibels true peak |
| DNA | Deoxyribonucleic acid |
| dpf | Days post fertilisation |
| DWC | Dilution water control |
| EDF | Extended depth of focus |
| ENU | *N*-ethyl-*N*-nitrosourea |
| FDA | Food and Drug Administration |
| FRET | Fluorescence resonance energy transfer |
| GCP | Good clinical practice |
| GFP | Green fluorescent protein |
| HEI-OC1 | House Ear Institute Organ of Corti 1 |
| hpf | Hours post fertilisation |
| HTS | High throughput screening |
| Hz | Hertz |
| $IC_{50}$ | Half maximal inhibitory concentration |
| IHC | Inner hair cell |
| ISI | Inter-stimulus interval |
| JNK | C-Jun N-terminal kinase |
| LC/MS | liquid chromatography mass spectrometry |
| LILO | Lead identification lead optimisation |
| LL | Lateral line |
| LLC | Long-latency C-start |
| LOEC | Lowest observed effect concentration |
| mRNA | messanger RNA |
| MTC | Maximum tolerated concentration |
| MTD | Maximum tolerated dose |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NADPH | nicotinamide adenine dinucleotide phosphate-oxidase |
| NCE | New chemical entity |
| NIH | National insitutes of health |
| NMDA | N-Methyl-D-aspartate |
| OCT | Organic Cation Transporter |

| | |
|---|---|
| OHC | Outer hair cell |
| OKR | Optokinetic response assay |
| OMR | Optomotor response assay |
| PBS | Phosphate buffered saline |
| PLL | Posterior lateral line |
| pPLL | Posterior lateral line primordium |
| PTW | Phosphate buffered saline with Tween 20 |
| RNA | Ribonucleic acid |
| RNID | Royal National Institute for Deaf People |
| ROI | Region of interest |
| ROS | Reactive oxygen species |
| SC | Solvent control |
| SEM | Standard error of the mean |
| SLC | Short-latency C-start |
| SNP | Single nucleotide polymorphism |
| SOP | Standard operating procedure |
| SPL | Sound pressure level |
| SR | Seeker response |
| STAT | Signal Transducer and Activator of Transcription |
| Tg | Transgenic/transgene |
| TRPV1 | transient receptor potential cation channel subfamily V member 1 |
| TUNEL | Terminal deoxynucleotidyl transferase dUTP nick end labeling |
| V | Volt |
| VAST | Vertebrate automated screening technology |
| VEMP | Vestibular-evoked myogenic potential |
| Vpp | Voltage peak to peak |
| WHO | World health organisation |
| wt | Wildtype |
| XIAP | X-linked inhibitor of apoptosis protein |

# Contents

# Table of Figures

# Table of Tables

# Chapter 1 General Introduction

## 1.1 Hearing Loss, Tinnitus and Balance Disorders

### 1.1.1 Hearing Loss

#### 1.1.1.1 Prevalence and impact

Hearing loss is the most prevalent sensory impairment, affecting more than 278 million individuals worldwide (WHO). It is estimated that 10 million, or 1 in every 6, people in the UK are deaf or hard of hearing (AHL, 2011a; Vio and Holme, 2005). Strikingly, a report from 2004 ranks hearing loss as the most common moderate to severe disability globally (Mathers et al., 2008). The Medical Research Council has predicted that hearing loss will increase by approximately 14% every ten years as the worldwide population ages; this has been equated to a total of 14.5 million UK sufferers by 2031 (AHL, 2011b). Concurrent with these statistics, adult-onset hearing loss is anticipated to feature in the top 10 disease burdens in the world by 2030, giving it a greater disease impact than diabetes and cataracts (Mathers and Loncar, 2006).

The annual cost of hearing loss to the UK economy through unemployment has been estimated at more than 13 billion pounds; even in times of economic prosperity, people with severe to profound deafness are four times less likely to be employed (RNID, 2007; Shield, 2006). This cost will almost certainly grow in the coming years as the worldwide demographic alters towards an 'ageing population' and the incidence of leisure-based noise-induced hearing loss increases. Despite the rising economic costs associated with hearing loss, disproportionately little money is spent on its prevention; the money invested into research for individuals affected is close to 16-times less than for diabetes and 37-times less than for cardiovascular diseases (AHL, 2011b). The cost to the individual is also high, affecting sufferers both socially and psychologically. The congenitally deaf, or those undergoing paediatric-onset losses, may have problems with language acquisition, reading, writing and information processing. As a consequence, sufferers may experience social isolation, limitation of career choices and a dependence on others (Rutman, 1989). The impact of adult-onset hearing loss is somewhat different, with loneliness,

psychiatric disturbances and reliance on others more commonly experienced (Thomas and Herbst, 1980).

#### 1.1.1.2 Sensorineural hearing loss

Sensorineural hearing loss occurs as the result of damage to either the specialised hair cells within the cochlea (the auditory component of the inner ear) or to the auditory nerve (Cranial nerve VIII), and is predominantly associated with ageing. Around half of 61-80 year olds and 90% of the over 80s in the UK have some degree of hearing deterioration (AHL, 2011a; Davis, 1989; Shield, 2006). Although ageing is a major cause, approximately 40% of all cases are unrelated to age, with other causes including: prolonged exposure to loud noise, genetic predisposition, ototoxic medications, head injury and infectious diseases.

### 1.1.2 Tinnitus

Tinnitus is the perception of sound originating from the ear or head, without any obvious auditory input (Baguley, 2002). Following the onset of the condition, either continuous tinnitus or recurrent sporadic episodes of tinnitus can be experienced; these effects are commonly permanent but can also be reversible. The noises experienced are often described as ‘ringing in the ears’ and are characteristically high-pitched, sometimes with musical or mechanical properties. In the UK, approximately 1% of the population have tinnitus which affects the quality of everyday life and around 10% of adults experience mild to moderate tinnitus on a regular basis (Davis and Rafaie, 2000). There is no single underlying cause of tinnitus, but the symptom is more prevalent in the elderly and individuals with hearing impairment and experienced more often by women than men (Holmes and Padgham, 2011). Tinnitus can also be caused by prolonged exposure to loud noise, ototoxic medications, infection, altered blood pressure and even stress. Severe tinnitus can have long-term psychological impact, with individuals suffering from lack of concentration, troubled sleep, depression and irritation. The economic cost of tinnitus is not known.

### 1.1.3 Balance Disorders

Balance (vestibular) disorders occur as a consequence of damage to the labyrinth of the inner ear and/or the vestibulocochlear nerve, and can be caused

by a variety of factors including acute injury, infections, medications, genetic disorders and environmental conditions. Commonly, the balance difficulty is idiopathic. Symptoms can include imbalance, vertigo, dizziness, hearing changes, blurred vision, nausea and coordination problems (VEDA, 2010). The prevalence of vestibular disorders is difficult to estimate; a 1998 survey entitled "Acceptability, Benefit & Costs of Early Screening for Hearing Disability" found that on average 22% of responders had experienced attacks of dizziness (Davis and Moorjani, 2002). The socioeconomic cost of balance disorders is not known.

## 1.2 Ototoxicity

### 1.2.1 Definition of Ototoxicity

Ototoxicity is defined as the cellular and functional damage to the hearing and/or balance systems of the inner ear or the vestibulocochlear nerve caused by substances, typically medicines and other chemicals. Ototoxicity may be permanent, causing lasting damage to the hearing or balance systems, or reversible. This distinction is dependent on the class of ototoxin and its specific pathophysiology. The principal symptoms of ototoxicity are hearing loss (initially at high frequencies), tinnitus and disequilibrium.

Ototoxicity is distinct from neurotoxicity resulting in hearing impairment as neurotoxins exert their damage in the brainstem and the cochlear and vestibular nuclei (Monsell, 2007).

### 1.2.2 Risk Factors

Multiple factors can increase an individual's susceptibility to adverse ototoxic effects. Contributing factors include age, dosing regimen and route of administration, concomitant administration of additional ototoxic substances, renal dysfunction and pre-existing hearing loss (Gratton and Smyth, 2004; Prepageran and Rutka, 2004; Rotstein and Mandell, 2004; Yancey et al., 2012).

Interestingly, certain genetic variants have been linked to ototoxicity. Single nucleotide polymorphisms (SNPs) in mitochondrial 12S rRNA (A1555G and Δ961Cn) that are linked to increased susceptibility to the ototoxic effects of aminoglycoside antibiotics have been discovered in many populations worldwide (Bacino et al., 1995; Fischel-Ghodsian et al., 1993; Fischel-

Ghodsian, 1999; Hamasaki and Rando, 1997; Prezant et al., 1993; Tang et al., 2002). Additionally, non-synonymous variants in *MT-ND3* and *MT-CYB* genes have also been identified as potentially harmful in the South African population (Human et al., 2010). Certain mutations in Glutathione S-Transferase (*GSTM3*B*, $GSTP_1$) have been proposed to protect from cisplatin-induced hearing loss, whereas other SNPs in the same gene (such as *GSTM1*) are potentially detrimental (Oldenburg et al., 2007a; Oldenburg et al., 2007b; Peters et al., 2000). Increased incidence of cisplatin-induced ototoxicity has also been linked to polymorphisms in a number of other genes, including *Megalin*, *Thiopurine S-methyltransferase* and *Catchol-O-methyltransferase* (Riedemann et al., 2008; Ross et al., 2009).

### 1.2.3 Common Classes of Ototoxin

Ototoxic drugs are commonly categorized by therapeutic class (See Table 1.1). Some of the most well known classes include the aminoglycoside antibiotics, platinum-based chemotherapeutics, salicylates, loop diuretics, and anti-malarials. Each ototoxin has its own associated effects on the ear and vestibulocochlear nerve. Effects may be reversible or irreversible and range in severity.

**Table 1.1:** A selection of medicines with known ototoxicity in humans.

| Drug class | Compound | Therapy | Disease |
|---|---|---|---|
| Aminoglycoside | Amikacin | Antibiotic | *Pseudomonas aeruginosa, Acinetobacter, Enterobacter* and tuberculosis. |
| | Gentamicin | | *Pseudomonas, Proteus, Serratia,* and the Gram-positive *Staphylococcus.* |
| | Kanamycin | | Obsolete. |
| | Neomycin | | *E. Coli, Enterobacter aerogenes, Klebsiella pneumoniae.* |
| | Netilmicin | | Similar activity to gentamicin, but less ototoxicity. |
| | Streptomycin | | Infective endocarditis, Tuberculosis, Plague (*Yersinia pestis*). |
| | Tobramycin | | *Pseudomonas.* |
| Cytotoxic Agents | Bleomycin | Chemotherapeutic | Hodgkin lymphoma, Non-Hodgkin lymphoma (NHL), Penile cancer, Squamous cell carcinoma of the (cervix, vulva, the head and neck), Testicular cancer. |
| | Carboplatin | | Ovarian carcinoma, lung, head and neck cancers. |
| | Cisplatin | | Bladder cancer, Cervical cancer, Malignant mesothelioma, Non-small cell lung cancer, Ovarian cancer, Squamous cell carcinoma of the head and neck, Testicular cancer. |
| | Nitrogen mustard | | Hodgkin's disease, non-Hodgkin's lymphoma, lung cancer, breast cancer, cutaneous T-cell lymphoma. |
| | Vinca alkaloids | | Leukaemia, rhabdomyosarcoma, neuroblastoma, Wilm's tumour, Hodgkin's disease, non-Hodgkin's lymphoma, breast cancer, and germ cell tumours. |
| Iron-Chelating agents | Deferoxamine (desferrioxamine B) | Chelator | Haemochromatosis and iron poisoning. |
| Loop Diuretics | Ethacrynic acid | Diuretic | Congestive cardiac failure, renal failure, cirrhosis, hypertension. |
| | Furosemide | | |

| | | | |
|---|---|---|---|
| Macrolides | Azithromycin | Antibiotic | Infections relating to HIV. |
| | Clarithromycin | | Activity against gram-positive and gram-negative infections, MAC microorganisms. |
| | Erythromycin | | Variety of infections including upper and lower respiratory tract infections. |
| Salicylates | Aspirin | Analgesic, antipyretic, anti-inflammatory | Fever, pain, inflammation, heart attack (follow-up treatment), colorectal cancer (prevention). |
| Anti-malarials | Quinine | Anti-Malarial | Prevention of malaria. |
| Topical agents | Chloramphenicol | Antibiotic | Eye and ear infections. |
| | Polymixin | | Infections of the skin, mucous membranes, eye and ear. |

#### 1.2.3.1 The Aminoglycoside Antibiotics

The aminoglycosides (aminoglycosidic aminocyclitols) are bactericidal antibiotics used primarily to treat aerobic gram negative infections. The first aminoglycosides were discovered in the 1940s, and were isolated actinomycete bacteria found in soil (Montie and Patamasucon, 1995).

All aminoglycosides have common chemical features, each possessing a single hexose nucleus, or aminocyclitol ring. In every class representative except streptomycin, this hexose nucleus is 2-deoxystreptamine, whereas in streptomycin, the hexose is streptidine. The aminocyclitol ring is additionally linked to two or more amino sugars via glycosidic bonds (Brunton et al., 2006; Figure 1.1).

The aminoglycosides exert their bactericidal effects by perforating the cell walls of target bacteria and inhibiting bacterial protein synthesis. The action of this class of antibiotic on gram negative bacilli can be explained by their natural charge; the aminoglycosides are positively charged and are thus attracted to the anionic lipopolysaccharides of the outer surface of the bacterial membranes, perforating them (Hancock and Bell, 1988; Peterson et al., 1985) . Once inside the bacterial cell, aminoglycosides interact specifically with the prokaryotic ribosomes in the small 30S and large 50S subunits (Campuzano et al., 1979; De Stasio et al., 1989; Fourmy et al., 1996; Fourmy et al., 1998; Misumi et al., 1978; Moazed and Noller, 1987; Scheunemann et al., 2010; Woodcock et al., 1991). The aminoglycosides can bind to the A-site of the bacterial ribosome's small subunit and cause tRNA to misread mRNA codons (Davies et al., 1965; Davies et al., 1966; Davies and Davis, 1968). An interaction can also occur between the small and large subunits of the ribosome, causing linking of two components and preventing recycling of the ribosome (Scheunemann et al., 2010). Misread protein produced by the binding of aminoglycosides may also be incorporated into membranes, causing abnormal channel formation and damage to the outer membrane of bacteria, leading to increased uptake of aminoglycosides and formation of a positive feedback loop (Busse et al., 1992; Davis, 1987; Montie and Patamasucon, 1995). The action of these antibiotics on bacteria is dose-dependent and persistent, even after withdrawal of treatment.

Aminoglycosides are administered in various forms: topically, intravenously, orally and intramuscularly (Joint Formulary Committee, 2011). The serious dose-limiting side-effects of the aminoglycosides are nephrotoxicity, neuromuscular blockade and ototoxicity (auditory and vestibular). The oto- and nephrotoxic effects of the aminoglycosides were noted early in their use, in some of the first clinical trials for tuberculosis (Hinshaw and Feldman, 1945). Although the exact incidence is uncertain, reviews of the literature in 2004 and 2011 estimated up to 47% ototoxicity in adults undergoing aminoglycoside treatment; these estimates were based on a variety of clinical studies and meta-analyses (Rotstein and Mandell, 2004; Xie et al., 2011). Individual risk factors such as cumulative dose, duration of treatment and combined therapy may

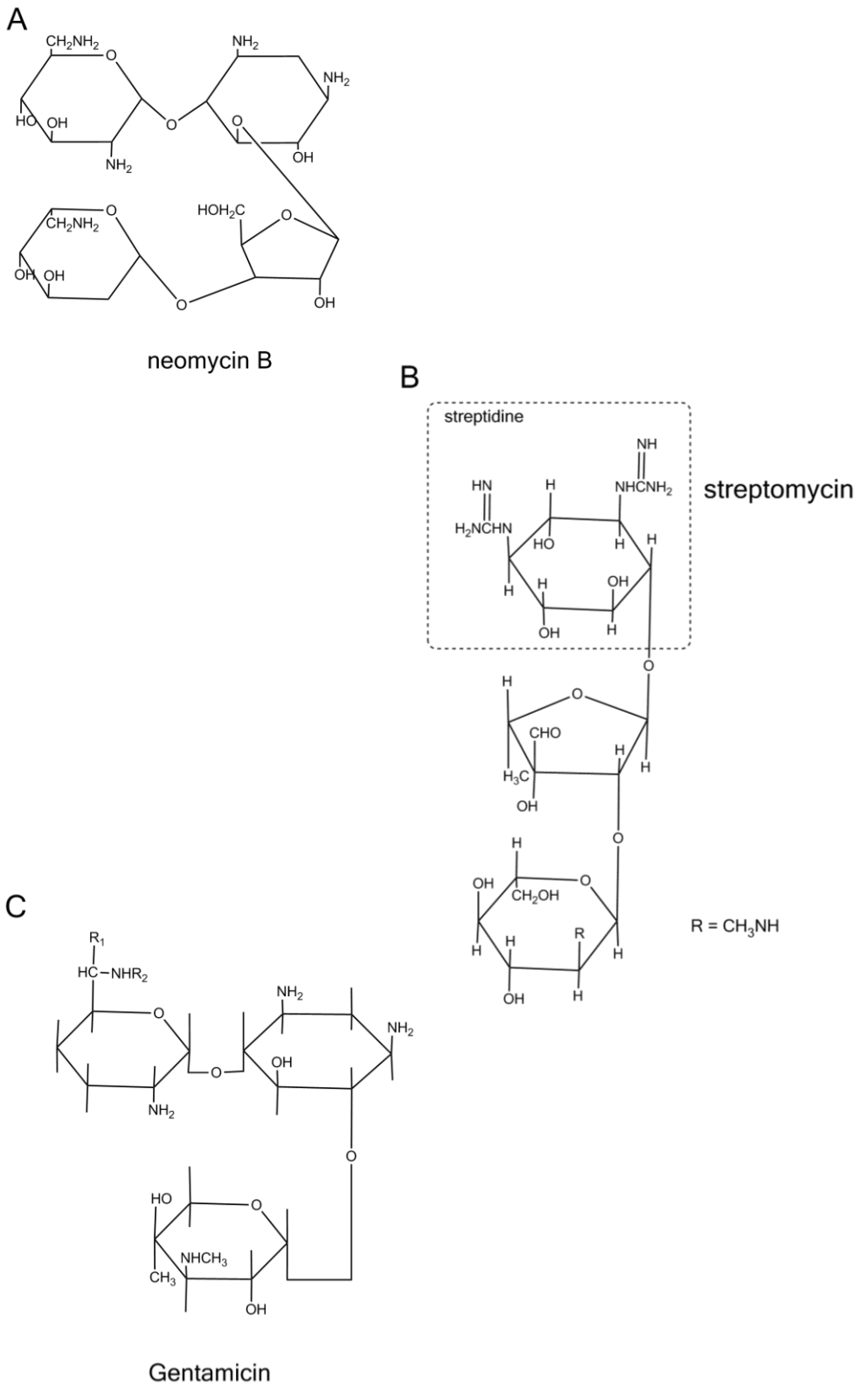



**Figure 1.1: The chemical structure of the aminoglycoside antibiotics.** Some examples of the aminoglycoside antibiotics. (A) Neomycin. (B) Streptomycin. (C) Gentamicin.

influence susceptibility to aminoglycosides in patient populations, although genetic susceptibility remains the only proven risk factor (Prezant et al., 1993; Xie et al., 2011).

Aminoglycosides preferentially accumulate in the renal cortex and in the endolymph and perilymph of the inner ear; their half lives are up to six times longer in the otic fluids than in blood plasma (Brunton et al., 2006; Voldrich, 1965). Resultant ototoxic damage occurs mainly at the level of the sensory hair cells in both the cochlea and vestibular apparatus. In some cases, degeneration of spiral ganglion neurons, nerve fibres and supporting cells and atrophy of the stria vascularis can be observed (Fischel-Ghodsian, 2004; Schacht, 2004). In the cochlea, damage occurs from the base to the apex causing high frequency hearing loss initially, resulting in irreversible auditory threshold shifts (Huizing and de Groot, 1987; Schacht, 2004; Xie et al., 2011). The outer hair cells appear to be more susceptible to the damage. Vestibular injury can manifest in nausea, vomiting, disequilibrium, vertigo and nystagmus. Different effects are observed depending on the specific compound administered.

Evidence from whole animal and cell line studies on the aminoglycosides has led to the suggestion of a variety of mechanisms of uptake and ototoxic damage over the years. Proposed mechanisms of uptake and accumulation of the aminoglycosides in hair cells include: receptor-mediated endocytosis, mechanotransduction and unconventional myosin-VII-A (Gale et al., 2001; Hashino and Shero, 1995; Hashino et al., 1997; Marcotti et al., 2005; Richardson et al., 1997; Williams et al., 1987). Uptake is not directly correlated to toxicity.

The ear-specific toxicity of the aminoglycosides could be attributed to a combination of selective uptake through ion channels due to positive endolymphatic potential and tissue specific intracellular binding targets, such as CLIMP-63 (Karasawa et al., 2008; Karasawa et al., 2010; Marcotti et al., 2005).

To date the most convincing explanations for the mechanisms of damage inside auditory cells are the stimulation of reactive oxygen (and possibly reactive nitrogen) species production by the formation of heavy metal-aminoglycoside complexes, and polyphosphoinositide lipid-aminoglycoside binding, leading to

generation of lipid peroxides (Clerici et al., 1996; Hirose et al., 1997; Hong et al., 2006; Karasawa and Steyger, 2011; Lodhi et al., 1980; Priuska and Schacht, 1995; Priuska et al., 1998; Sha and Schacht, 1999a; Sha and Schacht, 1999b; Sha et al., 2001a; Takumida and Anniko, 2002). This is supported by studies displaying varying levels of otoprotection using either free radical scavengers, or iron chelators (Conlon et al., 1998; Conlon and Smith, 1998; Conlon et al., 1999; Dehne et al., 2002; Garetz et al., 1994; McFadden et al., 2003; Sha et al., 2001b; Song and Schacht, 1996; Song et al., 1997).

Most reports of cell death induced by aminoglycosides suggest apoptosis is the main mechanism of hair cell death, but some necrosis has also been observed (Forge and Li, 2000; Jiang et al., 2006; Nakagawa et al., 1998). Various studies have shown evidence for apoptosis in hair cells and spiral ganglion cells, including JNK (c-Jun N-terminal kinase) and caspase activation, increased TUNEL staining, phosphatidylserine externalisation at the apical membrane and extrusion of hair cells from the organ of Corti (Alam et al., 2007; Bae et al., 2008; Cheng et al., 2003; Cunningham et al., 2002; Goodyear et al., 2008; Hirose et al., 2004; Jeong et al., 2010; Jiang et al., 2005; Kil et al., 1997; Lang and Liu, 1997; Lee et al., 2004a; Li et al., 1995; Nakagawa et al., 1997; Nakagawa et al., 1998; Sugahara et al., 2006; Taylor et al., 2008). Further to this, there is evidence that inhibition of caspases and JNK signalling and manipulation of apoptotic mediators such as XIAP (X-linked inhibitor of apoptosis protein) can partially protect hair cells from ototoxic insult (Forge and Li, 2000; Matsui et al., 2003; Nakamagoe et al., 2010; Okuda et al., 2005; Pfannenstiel et al., 2009; Pirvola et al., 2000; Tabuchi et al., 2007; Wang et al., 2003b; Wei et al., 2005; Ylikoski et al., 2002).

#### 1.2.3.2 Platinum coordination complexes

Platinum-based chemotherapeutics are a sub-class of alkylating agents frequently used to treat a wide range of aggressive cancers from germ-cell tumours of the testes, to lung cancer (Joint Formulary Committee, 2011; Sturgeon, 2004). Their chemotherapeutic potential was first identified in the mid 1960s by Rosenberg and colleagues (Rosenberg et al., 1965; Rosenberg et al., 1969). The compounds cisplatin, carboplatin and oxaliplatin are all platinum coordination complexes (Figure 1.2).

These compounds can bind covalently to nucleophilic sites on DNA such as the N7 of guanine, causing intra- and inter-strand cross-linking. These DNA adducts prevent the proliferating cell from carrying out transcription and replication, resulting in DNA strand breaks, miscoding and ultimately apoptosis (Brunton et al., 2006).

The dose-limiting side-effects of the platinum chemotherapeutics are peripheral neuropathy, nephrotoxicity, reversible myelosuppression and ototoxicity (Brunton et al., 2006).

Like the aminoglycosides, platinum-based chemotherapeutics cause permanent high frequency hearing loss after repeated dosing, with preferential loss of the outer hair cells of the basal turn of the cochlea. Damage has also been observed at the level of the stria vascularis, the vestibulocochlear nerve and in the spiral ganglion cells. Additionally, vestibular perturbation and tinnitus have been reported in some patients (Aguilar-Markulis et al., 1981; Boheim and Bichler, 1985; Brunton et al., 2006; Gratton and Smyth, 2004; Schaefer et al., 1985; Wright and Schaefer, 1982).

Despite its ototoxic effects, cisplatin is one of the most effective therapies for solid tumours and therefore the seriousness of any side-effects have to be weighed against the drug's high therapeutic value.

The overall ototoxicity of cisplatin has been estimated by one review of patient case reports as between 4 and 91% (Fausti et al., 1984). There are a number of reasons for this highly variable estimate. In particular, differing methods of ototoxic assessment and individual susceptibility factors can have an impact on the ototoxic outcome. Cumulative dose and age are predictors of cisplatin ototoxicity with children especially prone to its ototoxic effects (Weatherly et al., 1991; Yancey et al., 2012).

Cisplatin is a square-planar inorganic platinum molecule consisting of a central platinum element incorporating *cis*-positioned pairs of chlorine atoms or amine groups. The ear-specific toxicity of cisplatin in higher vertebrates could be attributed to the selective uptake of cisplatin into cochlear tissues either via organic cation transporter 2 (OCT2), or copper transporter 1 (Ctr1). In *OCT1/2*$^{-/-}$ knockout mice, cisplatin does not cause ototoxicity. Furthermore, inhibition of OCT2 with cimetidine protects against cisplatin ototoxicity (Ciarimboli et al., 2010). On the other hand, small interfering RNA against the *Ctr1* gene decreases cisplatin uptake in HEI-OC1 cells in vitro. Additionally, competitive inhibition at the level of the Ctr1 channel with copper sulphate decreases the uptake and toxicity of cisplatin in vitro and in mice in vivo (More et al., 2010).

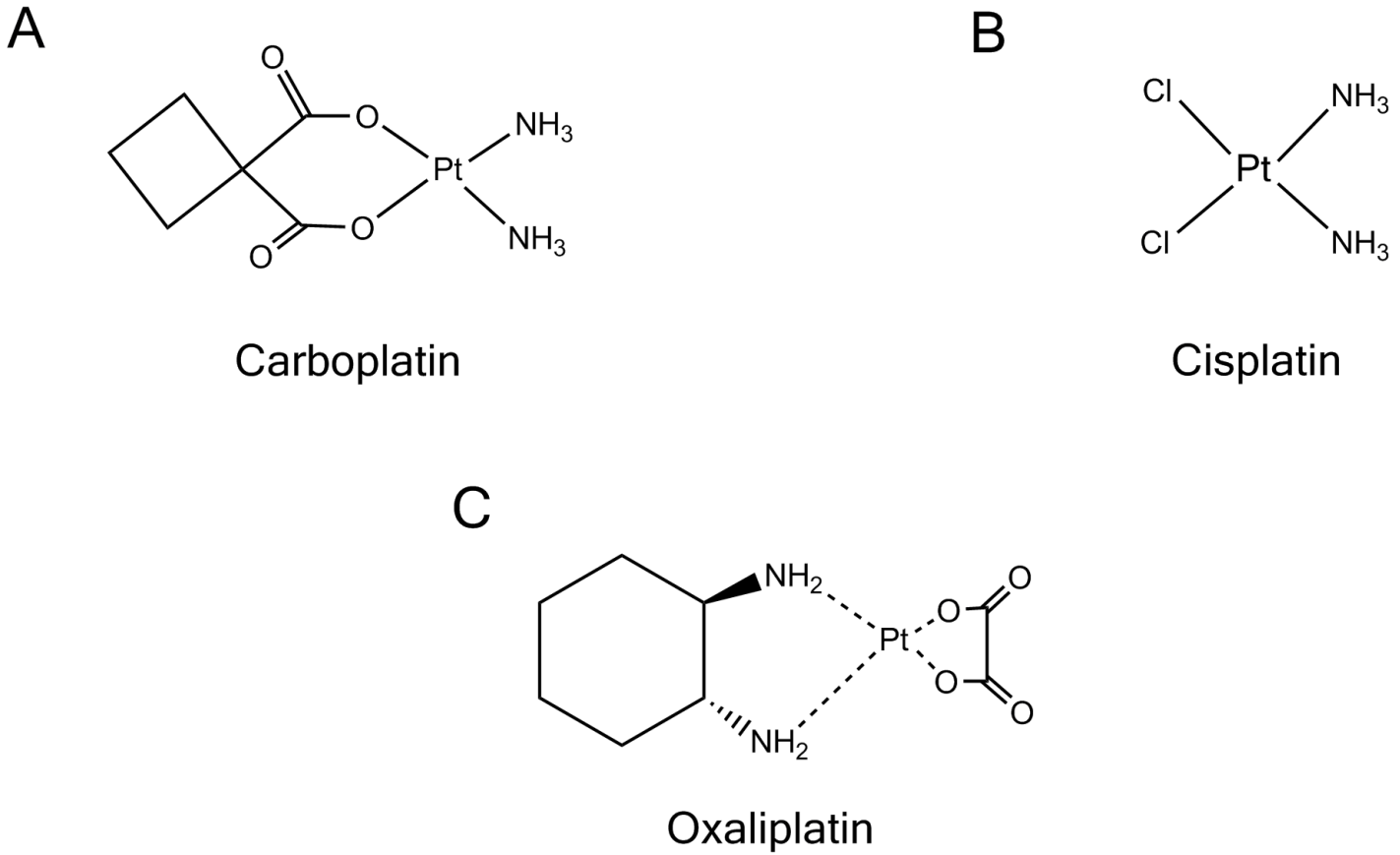



**Figure 1.2: The chemical structures of the platinum chemotherapeutics.** (A) Carboplatin, (B) Cisplatin and (C) Oxaliplatin.

Numerous studies suggest that cisplatin causes damage to hair cells via the generation of reactive oxygen species (ROS) (reviewed by Rybak et al., 2009). The increase in reactive oxygen species is thought to be triggered by induction or activation of the NADPH oxidase family of enzymes and TRPV1 (transient receptor potential vanilloid 1) ion channels (Mukherjea et al., 2008; Mukherjea et al., 2010). Increases in ROS subsequently lead to downstream apoptotic and necrotic events. Some of these downstream events (such as induction of pro-inflammatory factors, p53 and pro-apoptotic factors) are known to be mediated by the activation of STAT transcription factors (Kaur et al., 2011; Schmitt et al., 2009). The mechanisms of cisplatin-induced hair cell death will be discussed in more detail in Chapter 6.

#### 1.2.3.3 Salicylates

Salicylates are commonly taken to treat fever, pain and inflammation. The best known salicylate, aspirin, exerts its therapeutic effects by inhibiting the cyclooxygenase enzymes COX-1 and COX-2 in a reversible manner (Vane, 1971; Vane and Botting, 2003; for compound structure, see Figure 1.3).

Aspirin and other salicylate derivatives have long been reported to have ototoxic effects. The first reports date back to 1877, with aspirin ototoxicity reported around the time of its synthesis by Bayer in 1899. Salicylate toxicity is reported to occur in around 11 per 1000 individuals (Prepageran and Rutka, 2004). Often the hearing loss associated with the salicylates is mild to moderate, bilateral and reversible and frequently concomitant with tinnitus (Day et al., 1989).

The exact sites of aspirin-induced damage in the ear are debated, as there is conflicting histological evidence, both from humans and from non-human animal models. Pathological alterations have been reported in the organ of Corti, stria vascularis, spiral ganglion and outer hair cells (Tuper et al., 2005).

There are many current theories concerning the underlying mechanisms of aspirin-induced damage. Acoustic emission studies indicate that outer hair cell abnormalities are important in hearing loss. Decreased cochlear blood flow may also contribute to hearing loss; this has been associated with catecholamines and arachidonic acid metabolism (Didier et al., 1993) . A more recent finding is that the dissociated ion of salicylate competes with chloride ions to couple to the

anion-binding site associated with prestin (a voltage-sensitive membrane protein responsible for outer hair cell (OHC) electromotility). This interaction inhibits the action of prestin, thus limiting electromotility and cochlear amplification (Chen et al., 2010; Dallos, 2008; Kakehata and Santos-Sacchi, 1996; Oliver et al., 2001).

One proposed mechanism for aspirin-induced tinnitus is that altered levels of arachidonic acid increase the open probability of the NMDA receptors, thus activating them. The increase in NMDA receptor activity could lead to excitotoxicity (Guitton et al., 2003; Puel and Guitton, 2007).

#### 1.2.3.4 Loop Diuretics

Loop diuretics are a subclass of diuretics administered orally or intravenously to treat renal failure, cardiac failure, hypertension, cirrhosis and also bronchopulmonary dysplasia in premature infants (Prepageran et al., 2004). The loop diuretics act to block the reabsorption of sodium and chloride in the loop of Henle and proximal renal tubule of the kidney (Ikeda et al., 1997; Rybak, 1993).

The ototoxicity of loop diuretics such as ethacrynic acid and furosemide is well documented (for structures, see Figure 1.3), with the earliest reports occurring in the 1960s and 1970s (Maher and Schreiner, 1965; Rybak, 1988; Schwartz et al., 1970). The ototoxicity tends manifest in reversible sensorineural hearing loss, although occasional reports of permanent hearing loss have been linked to patients with renal failure. Tinnitus and vertigo have also been recorded (Prepageran et al., 2004).

The main site of damage in the ear is the stria vascularis, with oedema and cystic changes observed. Very occasionally, damage to the outer hair cells has been reported in the basal turn of the cochlea (Arnold et al., 1981). Studies have suggested that the loop diuretics exert their ototoxicity by inhibiting ion channels in a manner similar to their effect in the kidney, and that this alters perilymph and endolymph composition. These alterations in fluid homeostasis are correlated with temporary reductions in the endocochlear potential (Ikeda et al., 1997; Prepageran et al., 2004).

#### 1.2.3.5 Anti-malarials

It has been well reported that a small number of anti-malarial drugs have ototoxic effects. In particular, cases of ototoxicity have been linked with the use of quinine. This was first reported in 1692 (Tange et al., 1997). The effects of quinine are reversible hearing loss, tinnitus and vertigo, although the mechanisms of damage are not well understood (reviewed in Gurkov et al., 2008; for compound structure, see Figure 1.3).

### 1.2.4 The Prevalence of Ototoxicity

The prevalence of ototoxicity is extremely difficult to estimate. There are many reasons for this.

Firstly, most data for the ototoxicity of marketed and environmental chemicals is based on individual patient or small cohort case studies and anecdotal evidence. The lack of a clear definition of an ototoxin is also a major barrier to clinical diagnosis, particularly as the extent of damage to hearing and balance function required for a substance to be classed as ototoxic has not been agreed (Konrad-Martin, 2005; Scott and Griffiths, 1994). The delayed onset of ototoxicity (up to 6 months for neomycin) and the continuing progress of the pathology after cessation of treatment are also limiting factors in diagnosis. Furthermore, the variability in the pathophysiological effects of the different ototoxins and the reversible nature of many compounds makes an ototoxic event hard to capture and define. Perhaps most surprisingly, standardised protocols for monitoring ototoxicity in the clinic are non-existent. According to the American Academy of Audiology, there are "...significant gaps in knowledge that preclude the formulation of a standard of practice in this area..." (AAA, 2009).

It is important to highlight that the prevalence of ototoxicity is likely higher in less economically developed countries and in the Far East, where known ototoxins such as the aminoglycoside antibiotics are frequently used as first line medications for common infections such as bronchitis, often due to their comparative low cost (Anon, 1994; Fischel-Ghodsian, 2004). This, combined with genetic predisposition to ototoxicity, makes the prevalence much higher in countries such as China, for example (Hu et al., 1991). One study in China found that out of 763 deaf-mutes, 167 cases had acquired deaf-mutism through aminoglycoside treatment (22%). Of these 22%, 47 cases had at least one relative with aminoglycoside-induced hearing impairment, suggesting genetic susceptibility (Hu et al., 1991).

From all of this, it is clear that the incidence of ototoxicity is likely to be underestimated and that those cases observed in the clinic are merely the 'tip of the iceberg'.

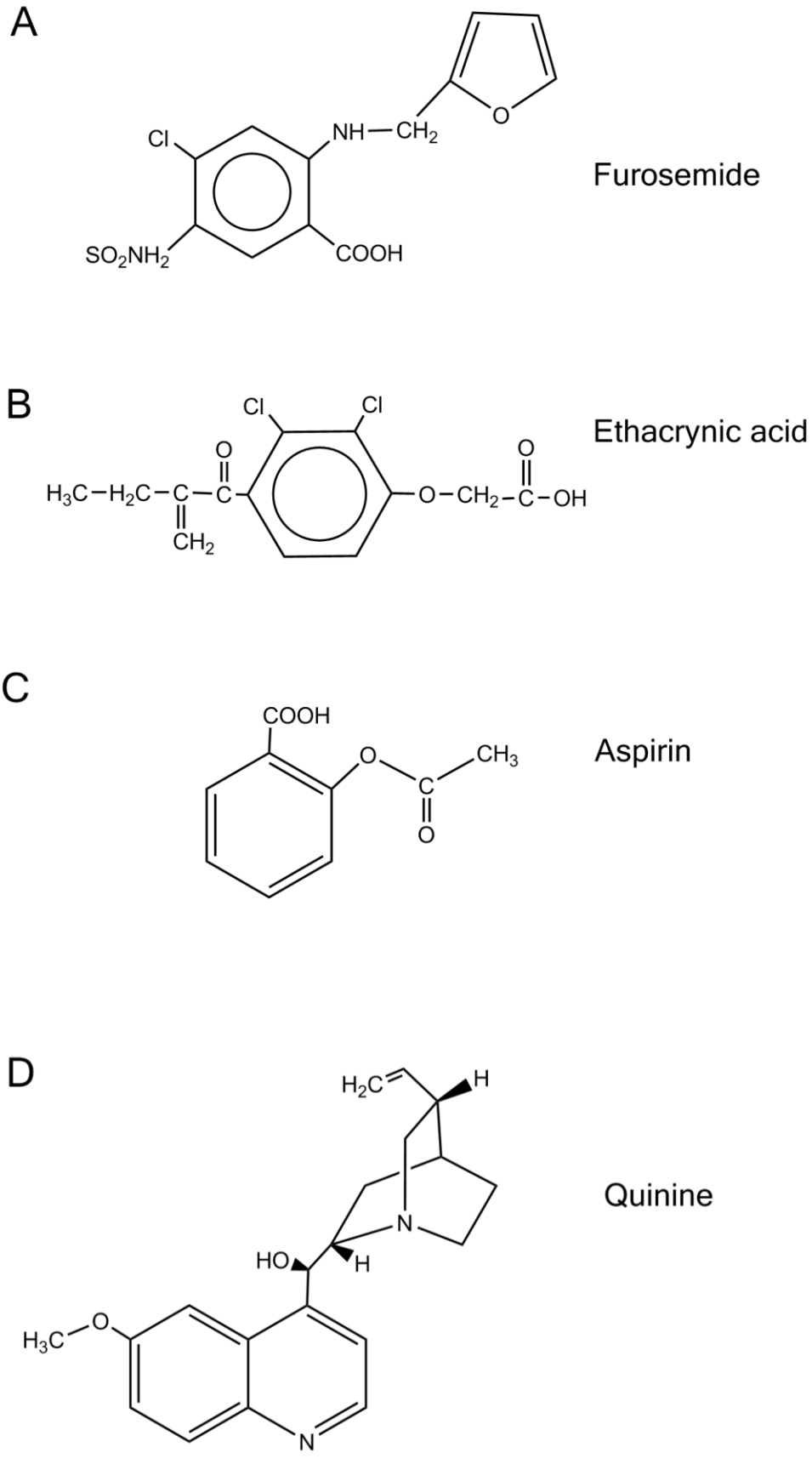



**Figure 1.3: The chemical structure of some ototoxins.** The loop diuretics furosemide (A) and Ethacrynic acid (B). (C) The salicylate derivative aspirin. (D) The anti-malarial quinine.

## 1.3 The Requirement for Ototoxicity Screening in Humans

Currently, the Food and Drug Administration (FDA) do not require ototoxicity screening in either the preclinical or clinical testing stages of drug development, and there are no Good Clinical Practice (GCP) guidelines in place. The exception to this is that monitoring is recommended at the clinical stage if the investigational compound is a member of a class of chemicals already recognised as ototoxic (AAA, 2009; Anon, 2010)

Similarly, ICH[1] guidelines call for a core battery of cardiovascular, central nervous system and respiratory testing in nonclinical safety pharmacology studies for human pharmaceuticals, but do not include auditory testing. As with the FDA, monitoring for adverse auditory effects using 'follow-up' safety pharmacology tests is only advised if the effects are expected (ICH, 2005).

Despite the lack of test guidelines currently in place for so-called 'low priority' adverse effects such as ototoxicity, the general consensus among pharmaceutical companies is that early hazard detection adds value. Early 'screening test methods' for adverse effects are advantageous in that they help to prioritise new chemical entities (NCEs). They also provide critical insights into the potential toxicity in man, thus enhancing the safe progression of a compound onto the market (Anon, 2005; Redfern et al., 2002; Valentin and Hammond, 2008).

As highlighted in section 1.2.4, ototoxicity is an underestimated problem with potential socioeconomic impact that is highly preventable. By employing early frontloaded screening during the lead identification and lead optimisation (LILO) stages as standard, the burden of ototoxicity has the potential to be greatly reduced.

## 1.4 Methods to Assess the Ototoxic Effects of Medicines

### 1.4.1 In vitro

Research into the ototoxic effects of compounds is performed in vitro using either organotypic culture, or less complex cell culture models.

[1] ICH = International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

#### 1.4.1.1 Organotypic culture

The earliest in vitro models of ototoxicity used explants of cochlear and vestibular tissues to model the damaging effects of compounds (Anniko et al., 1982; Richardson and Russell, 1991). This method is still employed by many laboratories and is often used in combination with in vivo tests of auditory function (Bas et al., 2012; Mazurek et al., 2012; Waissbluth et al., 2012). Most commonly, explants are taken from mouse, rat, guinea pig and chick tissues (Ding et al., 2011; Schmitt et al., 2009; Slattery and Warchol, 2010; Sly et al., 2012). As well as being able to combine organotypic culture experiments with in vivo functional assays, this in vitro method has additional advantages. The system allows for the investigation of the cellular mechanisms of drug-induced damage, regeneration and otoprotection in a 3D milieu also containing support cells. The major disadvantage of organotypic culture is its complexity. Often cochlear tissues are difficult to access, as the temporal bone must be removed from the animal and dissected. In some cases, dissection itself can damage the tissues. The method is easier for vestibular structures but is still relatively time-consuming.

#### 1.4.1.2 Cell culture

In the last 15 years, a number of cell lines have been created that are derived from inner ear structures. The majority of these cell lines are immortalised and take advantage of the Immortomouse model; in this transgenic mouse line, SV40 T antigen can be conditionally induced by temperature (Jat and Sharp, 1989; Jat et al., 1991). Specifically, a number of Organ of Corti (UB/OC-1, HEI-OC1, OC-k3) and vestibular (UB/UE-1, HEI-Ve1) derived lines have been established, which have been used to better examine the cellular mechanisms of ototoxicity and the potential of compounds to confer protection to hair cells (Chen et al., 2012; Kalinec et al., 1999; Lawlor et al., 1999; Park et al., 2012; Rivolta et al., 1998; Rivolta and Holley, 2002). The major advantage of this method of ototoxicity assessment is the high throughput and ease of experimentation compared with traditional in vivo and organotypic approaches. For example, this technique does not require the time-consuming isolation of cells required for organotypic culture. An obvious drawback is that the cells lack

the complex environment that is present in an in vivo system, reducing the translational capacity of the data generated.

### 1.4.2 In vivo

In vivo models of ototoxicity are mainly focussed on the *function* of the hearing and vestibular systems. The major advantage of in vivo studies is that cells remain intact and within their normal physiological environment, and the functional output allows a direct indication of potential clinical impact. The main disadvantages of in vivo studies are that the cellular mechanisms of damage are more difficult to investigate, experiments are expensive and compound requirements are high. This means that traditionally, if undertaken, in vivo audiometry can only be conducted towards the later stages of the drug development process. Additionally some animals such as mice are notorious for their variable responses to ototoxic insult, making the task of assessing ototoxicity, and translation to clinical risk, even more difficult (as highlighted in Ou et al., 2010).

#### 1.4.2.1 Auditory and vestibular testing

One useful method to test the auditory function in live animals is Auditory Brainstem Response (ABR) testing. In ABR testing, electrophysiological readings are recorded in response to direct auditory stimulation in the test ear (usually incremental tones).

Methods of vestibular monitoring are Vestibular Evoked Myogenic Potential (VEMP) and caloric testing. In VEMP testing, the myogenic potential of muscles in response to vestibular input is recorded. In caloric testing, directed nystagmus is induced and recorded by irrigating the auditory canal of the test ear with water of varying temperatures (Yang et al., 2010).

Functional assays such as these can be performed in a variety of animals from guinea pigs to chinchillas. The measurements taken are similar to those taken in human subjects and provide an excellent indication of ototoxic damage induced by either topical or systemic administration. An inherent advantage of the methods is that measurements taken before ototoxin exposure provide a direct comparison for each animal. Other major advantages of the techniques are that they can be performed in combination with post-mortem examination of

the tissues, for example electron microscopy and immunohistochemistry, and that repeated dosing can be performed. The shortcoming of the techniques is that they are not scalable; the requirement for high numbers of animals would be too costly and unethical (Yorgason et al., 2011).

#### 1.4.2.2 Knockout models

In recent years, mouse knock-out models have been created to study a selection of the proposed mechanisms of ototoxicity.

For example, knockouts have been used to great effect to study the mechanisms of cisplatin ototoxicity. *STAT1, STAT4* and *STAT6* knockout mice have been generated to investigate the role of the STAT pathway in the generation of pro-inflammatory cytokines in cisplatin-mediated hair cell death; it has been demonstrated that STAT1 and STAT6 signalling is required for ototoxicity (Kim et al., 2011; Schmitt et al., 2009). The role of OCT2 in the uptake of cisplatin has also been investigated using knockouts. Double knockout mice lacking *OCT1* and *OCT2* genes are partially protected from the hearing loss and cellular damage induced by cisplatin treatment (Ciarimboli et al., 2010).

Although important for providing valuable information on the mechanisms of ototoxin-induced damage, knockout mice are expensive and can take in excess of six months to generate.

## 1.5 The Zebrafish as an Alternative for Pre-Clinical Screening

Clearly, additional models for investigating the effects of new drugs on the hearing and vestibular systems are needed.

Any proposed model must confer the advantages of the mammalian, avian and in vitro systems and still be cost-effective, reliable and show good predictivity (Fleming and Alderton, 2012; Redfern et al., 2008; Valentin and Hammond, 2008). This is where fish models of auditory structure and function, and specifically the zebrafish, may have a role to play.

### 1.5.1 The zebrafish

The Zebrafish (*Danio rerio*) is a species of tropical freshwater fish originating from the Indian subcontinent (Spence et al., 2008). It is a member of the diverse

Cyprinid family of fishes, in the ray-finned class (Actinopterygii, grade Teleostei).

Since the 1980s, the zebrafish has gained popularity in the scientific research arena. The advent of two large scale ENU mutagenesis screens in the mid-1990s put the zebrafish on the map as a model of vertebrate development, developmental genetics and disease. This resulted in the publication of a whole collection of papers in a special issue of the journal *Development* in 1996 (Volume 123). Since this time, efforts have been made to sequence the entire zebrafish genome; the current zebrafish genome assembly, Zv9, was released in July 2010 (Sanger, 2012; www.sanger.ac.uk/Projects/D_rerio/). Most recently, the potential value of the zebrafish in higher throughout compound screening, re-profiling and toxicology has been explored.

### 1.5.2 Advantages

The zebrafish confers a number of advantages over more traditional models used for drug safety assessment. Adult zebrafish are small in size and relatively easy to maintain, making them cheap to house compared with higher vertebrates. The genetic tractability and rapid development of the animals also makes the generation of transgenic animals more practicable, quick and relatively inexpensive. As an in vivo model, the whole physiology of the animal can be studied rather than cells in isolation.

A number of advantages are also apparent regarding their suitability for developing higher throughput assays to frontload drug safety assessment. Female adult zebrafish have high fecundity, producing a large number of eggs in regular cycles. The speed of development and transparency of the fish at embryo and larval stages, as well as the use of transgenic reporter lines, enables the visualisation of organ system growth, development and physiology in addition to overall animal mortality (Kimmel et al., 1995). From around 24 hours post fertilisation (hpf) zebrafish larvae exhibit a number of stereotyped behaviours that can be readily observed and possess most of the major organs systems that mammals have. Most encouragingly, zebrafish larvae are also known to be capable of at least some drug metabolism (Alderton et al., 2010; David et al., 2012; Jones et al., 2010; Jones et al., 2012). These factors

combined make zebrafish a more attractive proposition than isolated cell preparations (Diekmann and Hill, 2012). Consequently, there are many examples of assays and screens having been developed to address a wide range of disease phenotypes and safety assessment targets, many of which are summarised below.

### 1.5.3 Use in screening and disease modelling

In 1997, the zebrafish was officially recognised by the National Institutes of Health (NIH) as a model organism for the study of vertebrate development and disease (Lessman, 2011; NIH, 2012). Since this time, the zebrafish has garnered a significant amount of interest among pharmaceutical companies (Redfern et al., 2008; Valentin and Hammond, 2008). In particular, leading organisations such as Pfizer, Novartis and AstraZeneca have all developed in-house assays or embarked on collaborations involving the use of zebrafish to assess drug safety, efficacy and pharmacokinetics (Fleming and Alderton, 2012; also see Table 1.2).

**Table 1.2:** Example assays performed in zebrafish to identify adverse drug reactions (ADRs).

| Safety screen | Assay type | Comments | Study |
|---|---|---|---|
| Behavioural alterations | Visual motor response test. | 57/60 compounds produced a change in activity at either basal or challenge phases. | (Ali et al., 2012) |
| | Photomotor response, touch-evoked response. Behavioural fingerprinting. | Phenotypic clustering. | (Kokel et al., 2010) |
| | Rest/wake regulation. Locomotor assays. | 3968 unique compounds screened. 463/3968 altered behaviour compared to control animals. Phenotypic clustering can identify novel targets/compounds. | (Rihel et al., 2010) |
| Seizure liability | In situ hybridisation screen, locomotor assay. | PTZ plus compound library. 46/2000 compounds decreased *cfos* and locomotor activity. Potential anti-convulsants. | (Baxendale et al., 2012) |
| | Locomotor assay. | 25 compound validation set. 72% predictivity. | (Winter et al., 2008) |
| Heart rate modulation/ cardiotoxicity | Embryonic heart rate. | *Tg:cmlc2:GFP*. Automated analysis (95% scored). Three positive and three negative controls. | (Burns et al., 2005) |
| | Heartbeat regularity in peripheral blood vessels. | terfenadine decreased heart rate and caused AV blockage. Increase in rhythmicity index. | (Chan et al., 2009) |
| | Q-T prolongation by video assessment and custom software. | Bradycardia induced by nifedipine. 12 Q-T prolonging drugs identified. Morpholino to HERG mimics Q-T prolonging compounds. | (Langheinrich et al., 2003) |
| Addiction | Conditioned place preference (CPP) in mutant lines. | *dum* and *jpy* mutants display cocaine insensitivity. | (Darland and Dowling, 2001) |
| | CPP. | Measured the reinforcing properties of D-amphetamine in adults. | (Ninkovic and Bally-Cuif, 2006) |
| Visual function | ENU mutagenesis then Optomotor (OMR) and optokinetic (OKR) response testing. | Mutations in 41 genes identified that affect visual function. Mostly in photoreception. | (Muto et al., 2005) |
| | OMR and OKR tests. | 27 compound validation set. OMR: 13/19 – expected positive effect (visual toxins). 6/8 – expected negative result. Useful predictor | (Richards et al., 2008) |

| | | | |
|---|---|---|---|
| | | of ADRs on visual function. | |
| Anxiety | Thigmotaxis. | Edge preference (anxiety) increases with caffeine and decreases with diazepam. | (Richendrfer et al., 2012; Schnorr et al., 2012) |
| Gut motility | Gut contractions counted by eye. | 16 compound blinded validation. 6/10 compounds showed the expected change in gut motility. | (Berghmans et al., 2008) |
| Ototoxicity | Vital dye staining of neuromasts | 1040 FDA approved compounds and bioactives. Seven known ototoxins identified, 14 novel compounds potentially ototoxic. | (Chiu et al., 2008) |

Additionally, the zebrafish has been utilised for the study of a wide range of human diseases ranging from obesity to cancer (see Table 1.3; Jones et al., 2008; Li et al., 2012; Liu and Leach, 2011; Ridges et al., 2012; Tingaud-Sequeira et al., 2011).

**Table 1.3:** Examples of zebrafish disease modelling.

| Disease | Assay | Comments | Study |
|---|---|---|---|
| Polycystic kidney disease | Chemical modifier screen for two mutant phenotypes. | Custom library 115 compounds. 6/115 altered body curvature. 14/115 affected asymmetry. | (Cao et al., 2009) |
| Acute renal failure/renal defects/diabetes mellitus | Injection of drugs into cardiac venous sinus. Measurement of clearance and histology. | Decreased renal clearance after treatment with gentamicin and cisplatin. Changes to pronephric tubules consistent with observations in mammals. | (Hentschel et al., 2005) |
| | Morpholino and dextran-toxin injection. | Morpholinos targeting CD2AP or podocin and puromycin aminonucleoside cause oedema and increased clearance. | (Hentschel et al., 2007) |
| Inflammation | Tail transection model and cell counts in *mpo:GFP* larvae. | Inflammation proceeds in a similar fashion (similar kinetics) to mammals. Neutrophil resolution can be manipulated by a caspase inhibitor. | (Renshaw et al., 2006) |
| | Chemically induced inflammation assay. Neuromasts and leukocytes visualised using transgenic lines. | 10/11 anti-inflammatory agents and ROS inhibitors inhibited leukocyte recruitment. Automated detection. Migratory impairment observed in *Was* mutant. | (d'Alençon et al., 2010) |
| | Pharmacological manipulation of resolution of inflammation. Measured by neutrophil counts using *mpo:GFP* line. | Caspase inhibitors delayed resolution. Bacterial LPS and dbcAMP also perturbed inflammation resolution. Polycyanin reduced neutrophil numbers in a caspase-dependent manner. Roscovitine accelerated resolution. 12/960 accelerated resolution of inflammation. | (Loynes et al., 2010) |
| Bacterial Infection | *S. aureus* infection into yolk or bloodstream. Survival curves. | Infection model established in wild type. Myeloid cells key in mounting infection response. Pu.1 knockdown larvae die more rapidly and cannot contain the infection. | (Prajsnar et al., 2008) |
| Atherosclerosis | Measuring vascular accumulation of MDA-LDL using *hsp70:IK17-EGFP* line. | Treatment with high cholesterol diet leads to increased accumulation of IK17 epitopes. Regression diet and antioxidant probucol leads to decreased IK17 accumulation. | (Fang et al., 2011) |

| | | | |
|---|---|---|---|
| Angiogenesis | Cognition Network Technology to look at intersegmental vessels. *Tg(fli1:EGFP).* | Intersegmental area and length are decreased significantly by the known anti-angiogenic agent SU4312. Microtubule inhibiting agents inhibit growth of intersegmental vessels. | (Vogt et al., 2009) |
| Obesity | Rate yolk sack diminished measured by Nile Red stain. | PPARγ receptor agonists cause increase in fat. Resveratrol decreased fat. | (Jones et al., 2008) |
| Cancer | Screening for anti-cancer agents | 59 of 502 natural compounds induced apoptosis or activated the p53 pathway. 28 already known for anti-cancer properties. 21 agreed with cell based MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay. | (Li et al., 2012) |
| | Screen for T-cell elimination in thymus using the *p56lck*:EGFP zebrafish line (*lck*:EGFP). | 26 400 molecules screened. Identified lenaldekar which selectively eliminates T-cells without affecting other cell types. | (Ridges et al., 2012) |

## 1.6 The Zebrafish auditory and vestibular system

Zebrafish use their acoustico-lateralis system, swim bladder and Weberian ossicles in combination to detect stimuli from the external environment; they are hearing specialists (Fay and Popper, 1974). The zebrafish acousticolateralis system is comprised of two key sensory structures, the inner ear (the utricular, saccular and lagenar maculae along with the semicircular canals) and the lateral line (Bever and Fekete, 2002; Ghysen and Dambly-Chaudière, 2004; Metcalfe et al., 1985; Metcalfe, 1989; Raible and Kruse, 2000; Whitfield et al., 2002). The acoustico-lateralis system has mixed auditory and vestibular function.

### 1.6.1 The Zebrafish Ear

The zebrafish inner ear system possesses remarkably similar functional capabilities to mammalian hearing systems, despite lacking a cochlea.

#### 1.6.1.1 Structure and development

The zebrafish inner ear forms from the otic placode, a transient thickening of ectoderm that can be observed in proximity to the caudal hindbrain from around 16 hpf (Ladher et al., 2010; Whitfield et al., 2002). The placode cavitates to form a vesicle approximately two hours later and by 24 hpf this vesicle contains the rudimentary sensory patches and otoliths. Between 22 and 42 hours post fertilisation, a subset of cells of the otic vesicle delaminate to become neuronal precursors; these form the statoacoustic ganglion (Haddon and Lewis, 1996; Whitfield et al., 2002).

At the early larval stages (including 5 days post fertilization (dpf)), the zebrafish inner ear possesses two patches of sensory epithelium, one within the future utricle (utricular or anterior macula) and one occupying the future saccule (saccular or posterior macula), each with an overlying otolith. The maculae are bathed in fluid and contained in interlinked compartments, similar to in other vertebrate systems although less complex. The maculae contain support cells and hair cells of similar, but not identical, structure and function to those seen in the mammalian and avian auditory systems. Each hair cell has a single apical kinocilium and a bundle of stereocilia that transduce signals via associated sensory neurons (Haddon and Lewis, 1996; see Figure 1.4).

As in all jawed vertebrates, zebrafish also possess three semicircular canals that are visible in larvae from approximately 3 dpf. Each canal contains small patches of hair cells in swellings or ampullae. The semicircular canals are formed by the joining of three pairs of cylindrical projections from the wall of the otocyst (Waterman and Bell, 1984).

In adult zebrafish, there are two additional sensory patches present, the lagena (hair cells visible at 13dpf) and macula neglecta. Sound waves are focused onto the saccule of the inner ear by the zebrafish swim bladder and Weberian ossicles (a system of small bones; described in Abbas and Whitfield, 2010).

#### 1.6.1.2 Function

The sensory maculae have auditory and/or vestibular function. The utricle is proposed to be primarily vestibular in function, detecting linear acceleration and gravity. The saccule has been proposed to have primarily auditory function, detecting sound. The semicircular canals are situated perpendicular to each other and detect position in relation to movement in a three-dimensional environment (Lambert et al., 2008). The sensory cells in the ampullae serve to detect this motion.

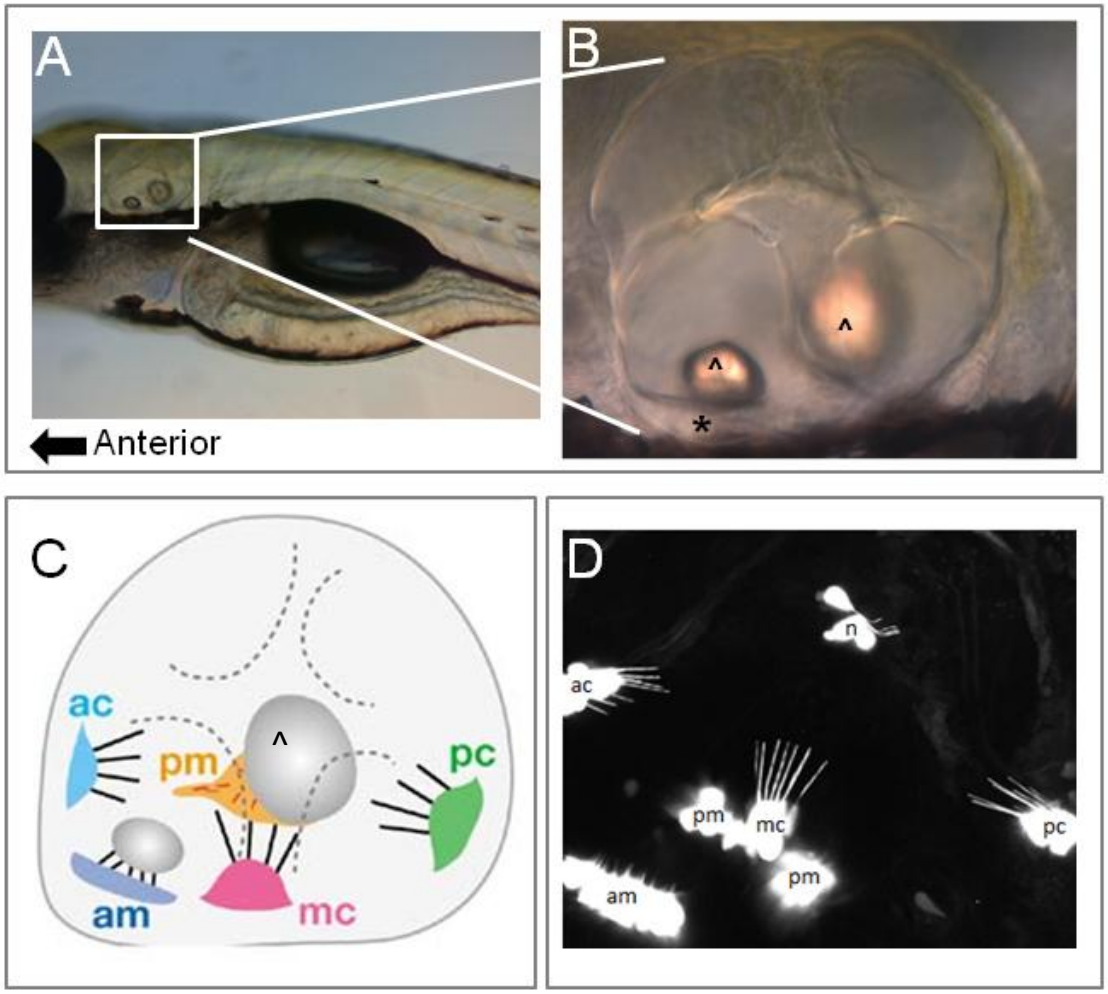



**Figure 1.4: The zebrafish ear at 5 dpf.** (A) and (B) Bright field images of the ear of a wild type larva at 5 dpf. Image is representative of wild-type larvae. The anterior macula (*), otoliths (^) and semi-circular canals can be observed. (C) Schematic of the larval ear displaying posterior ototlith (^), anterior macula (am), posterior macula (pm), anterior crista (ac), medial crista (mc) and posterior crista (pc). Diagram taken from Nicolson et al., 2005. (D) Confocal image of an ear from a *Tg*(*i193:GFP)* larva at 5 dpf (n is a cranial neuromast).

### 1.6.2 Lateral line

#### 1.6.2.1 Structure

The lateral line is a system of mechanosensory organs positioned around the head (anterior lateral line or ALL) and bilaterally along the horizontal myoseptum (posterior lateral line or PLL). These organs, called neuromasts, consist of sensory hair cells, mantle cells and support cells in a rosette-like formation (see Figure 1.5). The entire neuromast is surrounded by a gelatinous cupula.

The sensory hair cells are superficially located but embedded in the skin, protruding outwards. Each hair cell extends a ciliary bundle into the water. Vibrations through the water result in the displacement of hair cells and the transduction of a signal to the central nervous system. Mantle cells are accessory cells that line the neuromast; they are positioned so as to allow the kinocilia to protrude from a hole in the centre of the rosette. It is thought that the mantle cells are responsible for the production of the cupula and may aid in hair cell regeneration. Other supporting cell types lie in close proximity to the hair cells and are proposed to play a role in regeneration (Hernandez et al., 2007). Each neuromast is innervated by both afferent and efferent fibres which relay electrical signals to and from the brain respectively (Raible and Kruse, 2000).

The study described in this thesis mainly focuses on the posterior section of the lateral line at 5 dpf. At this stage in PLL development, there are usually nine mature neuromasts (on each side), with an extra two maturing neuromasts in an anterior position in line with the inflated swimbladder. The location of the neuromasts in the developing larva follows a stereotypical pattern (Gompel et al., 2001; see Figure 1.5).

.

#### 1.6.2.2 Development

In order to study the ototoxicity of compounds on the PLL, it is important to consider the complex development of the system.

The posterior lateral line develops from the lateral line placode, an epidermal thickening that originates from neural ectoderm. This placode lies caudal to the otic vesicle. At around 20 hpf, a posterior section of the placode detaches from the basement membrane and forms two divergent clusters of cells that are differentially controlled by cell signals. These separate clusters form the primordium and the ganglion (Ghysen and Dambly-Chaudière, 2004; Ghysen and Dambly-Chaudiere, 2007; Metcalfe et al., 1985).

The primordium of the posterior lateral line (pPLL) begins to migrate towards the tip of the tail at around 20 hpf and by approximately 48 hpf reaches its terminus, having deposited nine immature neuromasts on each side. These pro-neuromasts mature in a few hours to become mechanotransductively active (Murakami et al., 2003; Santos et al., 2006). As the primordium migrates, so too do the axons of the neurons that will innervate the mature neuromasts (Gilmour et al., 2004; Metcalfe, 1985). Myelination of the PLL nerve is subsequently performed by glial cells which travel along the axons (Brosamle and Halpern, 2002; Gilmour et al., 2002). Another wave of deposition occurs as a second primordium makes its way along a similar path.

The tightly controlled processes of primordium organisation, primordium migration, neuromast deposition and cell differentiation are coordinated by an elaborate combination of signalling pathways. These signals include the interaction of CXCR4 and CXCR7 with their SDF1 ligands to coordinate directed migration and neuromast deposition, and lateral inhibition from Notch/Delta to regulate cell specification within the neuromast (Dambly-Chaudiere et al., 2007; David et al., 2002; Haas and Gilmour, 2006; Itoh and Chitnis, 2001; Li et al., 2004; Sarrazin et al., 2006; Valentin et al., 2007). The FGF and Wnt/β-catenin pathways are crucial in many aspects of the development of the lateral line (Aman and Piotrowski, 2008; Gompel et al., 2001; Lecaudey et al., 2008; Nechiporuk and Raible, 2008).

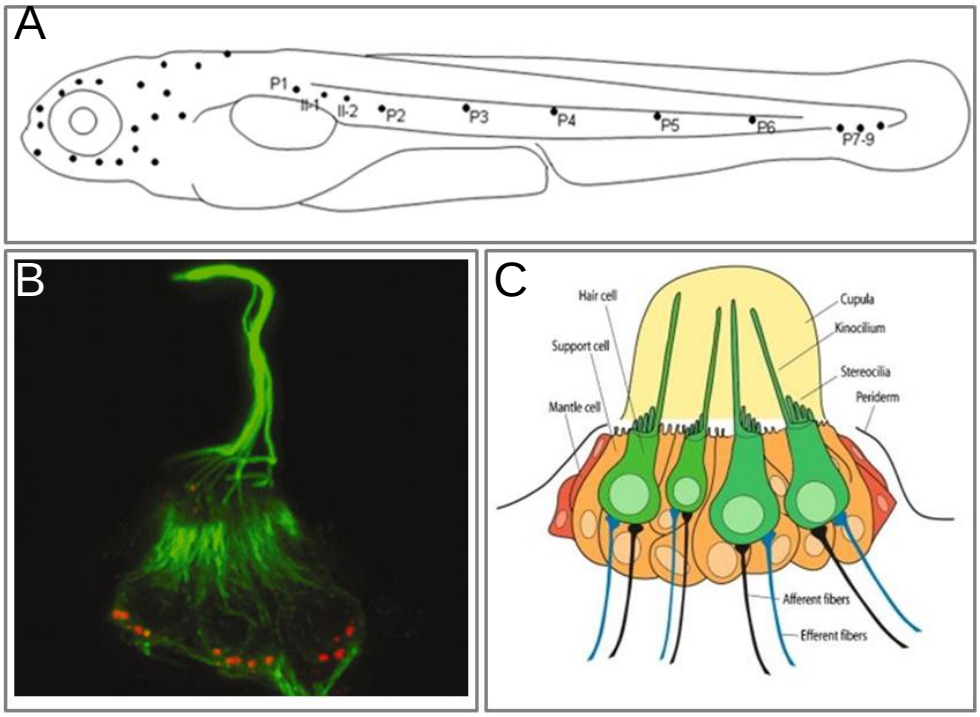



**Figure 1.5: The zebrafish lateral line.** (A) Schematic showing typical pattern of neuromasts in a 5 dpf zebrafish larva. Smaller neuromasts from the second wave of deposition are indicated (II-1, II-2). Taken from Buck et al., 2012. (B) Cross-section of a lateral-line neuromast at 4 dpf. Acetylated tubulin immunofluorescent labelling (green) shows innervating neurons and outlines the hair cells including kinocilia. Ribeye labelled puncta (red) localise to the basolateral end of the hair cells close to the neurons. Taken from Sheets et al., 2011. (C) Schematic of a neuromast showing hair cells, support cells, cupula and nerve supply (taken from Chiu et al., 2008).

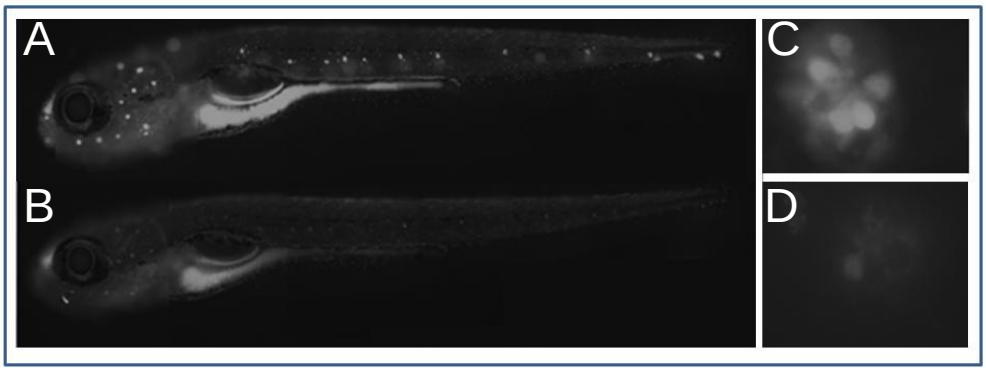



**Figure 1.6: Chemical screens for ototoxicity can be carried out using vital dyes.** (A) Control treated larva. The neuromasts of the lateral line are labelled with DASPEI (2-(4-(dimethylamino)styryl) -N-Ethylpyridinium Iodide). Staining in the nasal epithelium acts as an internal control. (B) Ototoxin treated embryo stained with DASPEI. Nasal epithelium staining can still be seen. (C) Close up of an untreated neuromast stained with DASPEI. (D) Close up of an ototoxin treated neuromast stained with DASPEI.

#### 1.6.2.3 Function

The lateral line senses changes in water flow, acting to perceive “touch-at-a-distance” (Hofer 1908 as reviewed by Dijkgraaf, 1963; Ghysen and Dambly-Chaudière, 2004; Kaus, 1987). It is capable of mediating a variety of important naturally occurring behaviours such as predator avoidance, feeding, station holding, obstacle detection and schooling (Coombs and Montgomery, 1999; Hassan, 1986; Montgomery et al., 1997; Partridge and Pitcher, 1980). Innate responses such as rheotaxis and the escape reflex are required to mediate these behaviours.

## 1.7 Use of zebrafish to model ototoxicity

In recent years, there has been considerable interest in the larval zebrafish as a model to investigate ototoxicity. This interested has stemmed from a number of important factors that make the zebrafish an appropriate model of auditory structure and function.

### 1.7.1 Genetic conservation

The conservation of genes involved in hearing function and in cell death pathways implicated in ototoxicity between the zebrafish and mammals makes it an excellent candidate species to study the effects of ototoxins.

Large scale mutagenesis screens have revealed numerous clinically significant inner ear and lateral line mutants (Nicolson et al., 1998; Schibler and Malicki, 2007; Whitfield et al., 1996). Subsequent characterisation of these mutants has resulted in the improved understanding of processes such as mechanotransduction and hair cell survival in the zebrafish, including similarities to the human disease state. For example, a candidate for a component of the transduction apparatus, the tip link, was discovered by analysis of the homozygous mutant *sputnik/cadherin 23* that completely lacks a tip link (Nicolson et al., 1998). Equally as impressive, the zebrafish *mariner* mutant is linked to a mutation in the gene that codes for Myosin VIIA. Mutations in the human *MYOVIIA* gene are known to cause hearing disorders including Usher 1B syndrome. Not only is this gene conserved, but mutants also show similar phenotypic and functional changes to *shaker-1* mice defective in the orthologous murine gene (Ernest et al., 2000; Gibson et al., 1995).

Multiple genes and pathways involved in apoptosis and oxidative stress are also conserved between zebrafish and higher vertebrates, facilitating the study of mechanisms of hearing loss induced by compounds. For example, cross-species functional conservation of the zebrafish and mammalian Bcl-2 apoptotic proteins, caspases and reactive oxygen-generating NADPH oxidase enzymes has been shown (Chakraborty et al., 2006; Jette et al., 2008; Kawahara et al., 2007; Niethammer et al., 2009).

### 1.7.2 Ototoxins

Numerous studies have demonstrated that human ototoxins can destroy or damage hair cells in the lateral line of larval zebrafish, although relatively few have focussed on effects in the inner ear (Giari et al., 2012; Seiler and Nicolson, 1999).

Many aminoglycosides including neomycin, streptomycin, gentamicin and kanamycin damage zebrafish hair cells in a dose-dependent manner (Seiler and Nicolson, 1999; Ton and Parng, 2005; Williams and Holder, 2000). It is now known that the actions of the aminoglycosides on hair cells are complex and involve early mitochondrial changes, calcium modulation, apoptotic and necrotic death events and different time-dependent phases of damage that potentially act via alternative signalling pathways (Coffin et al., 2009; Owens et al., 2007; Owens et al., 2009; Vlasits et al., 2012). Likewise, the chemotherapeutic agent, cisplatin, causes hair cell death in the zebrafish (Ton and Parng, 2005). The damage follows a much slower time course that continues after compound washout. Cisplatin is thought to exert its damage via both shared and divergent pathways compared to the aminoglycosides, including ROS generation (Choi et al., 2011; Kim et al., 2008; Ou et al., 2007; Owens et al., 2008; Vlasits et al., 2012; Wang et al., 2004). Additionally, the toxicity of heavy metals such as copper, zinc and iron has been demonstrated in the PLL, with attention being focussed on copper (Hernandez et al., 2006). Like aminoglycosides, copper is taken up into hair cells via mechanotransduction (Olivari et al., 2008). Once inside the neuromast it is capable of inducing ROS, inflammation and even damage to support cells at higher concentrations (d'Alençon et al., 2010; Hernandez et al., 2006; Linbo et al., 2006; Olivari et al., 2008).

Notably, the work of the Raible and Rubel laboratories has been of key importance in assessing the functional anatomy of the lateral line (Murakami et al., 2003; Raible and Kruse, 2000; Santos et al., 2006) and in the elucidation of drug-induced damage occurring in the hair cells of the neuromasts (Harris et al., 2003; Ou et al., 2007; Owens et al., 2007). More recently, the group's focus has shifted to an unbiased compound screening approach involving the identification of novel ototoxins, otoprotectants and modifiers of regeneration following ototoxic damage (Coffin et al., 2010; Ou et al., 2010). These studies build on the earlier work of Christopher Ton and Chuenlei Parng to bring the zebrafish to the forefront of ototoxicity research (Ton and Parng, 2005). Despite the shift to screening, the group still aims to investigate any "confirmed hits" further, in order to identify the mechanisms underlying their effects. Nowadays, promising "hits" from these screens are additionally assessed in cultured mouse utricles in vitro and more rarely, in live animals (Chiu et al., 2008; Ou et al., 2009; Owens et al., 2008).

### 1.7.3 Screens to identify ototoxins

Initially, zebrafish–based screens were designed to identify potential hair cell toxins among compounds in medical use in order to make clinicians aware of the danger (Figure 1.6).

The original screen focussed on detecting unknown ototoxic agents. A library of 1040 FDA-approved compounds and known bioactives was screened using vital dye techniques (NINDS custom library; Chiu et al., 2008). Twenty-one compounds were identified as potentially ototoxic in confirmatory retests. Of these, seven had known ototoxicity (emphasising the usefulness of the zebrafish in identifying known human ototoxins) and fourteen were proposed novel ototoxins (highlighting the potential of the zebrafish as a potential predictor of ototoxicity). Five false negatives were recorded when known human ototoxins were not detected. This was explained by the fact that assessment was only undertaken at one concentration for a single exposure time.

In the last year, a library of 88 anticancer drugs (National Cancer Institute Approved Oncology Drugs Set) was screened for ototoxic effects (Hirose et al., 2011). Unlike in the aforementioned screen, this assay was performed at

different concentrations and with two exposure durations. This time, 80% of previously reported ototoxins were identified correctly. Additionally, 57% of suspected human ototoxins were observed to cause hair cell damage and five new potentially ototoxic compounds were proposed (7% of the library).

### 1.7.4 Screens to identify otoprotectants

An alternative use for assays that are able to detect ototoxins is for the discovery of novel otoprotectants. There are two main reasons to identity putative otoprotectants. Firstly, pre-treating or co-treating with protectants could ameliorate the damage induced by ototoxins in humans. Secondly identifying the mode of action of protectants could help scientists to better understand the underlying mechanisms of the initial toxicity.

The first screen for protective compounds came in 2008. A library of 10 960 compounds was screened to identify molecules that protected hair cells of the lateral line from neomycin toxicity (Owens et al., 2008). This screen identified two compounds, the benzothiophene carboxamides PROTO1 and PROTO2, which exhibited robust protection across neomycin doses. Neither of the compounds inhibited aminoglycoside uptake, suggesting that they acted intracellularly to attenuate hair cell toxicity. In theory, these compounds could be used clinically to limit ototoxicity as they appeared not to interfere with the therapeutic action of neomycin. The screen also identified five protective mutants using an ENU mutagenesis screen and studied two (*persephone* and *sentinel)* in more detail. *Sentinel* is mutant in the *cc2d2a* gene, and is insensitive to aminoglycosides but still sensitive to cisplatin hair cell damage.

The most promising protectant discovered by screening so far is the Alzheimer's medicine tacrine. Tacrine was found in the NINDS library. It provided protection against neomycin-induced hair cell death in both zebrafish and in vitro in mouse utricles (Ou et al., 2009). As tacrine did not alter the bactericidal activity of neomycin and was already FDA-approved, it was an excellent candidate for in vivo validation and clinical testing as a potential otoprotectant (Coffin et al., 2010). On further testing of weaker "hits" from the original screen, eight quinoline-derived otoprotectants were also discovered (Ou

et al., 2009). These quinoline-derivates seem to exert their protective effects by inhibiting the uptake of the aminoglycosides (Ou et al., 2012).

The most recent screen from the Raible and Rubel laboratories looked for compounds that could protect against multiple ototoxins, including cisplatin and neomycin (Vlasits et al., 2012). This investigation highlighted the complexity of the ototoxins in terms of their different mechanisms of action. Ten compounds were found to protect hair cells from treatment with at least two of the four ototoxins tested. These came under four classes of drug: serotonin and dopamine modulating drugs, adrenergic receptor ligands and oestrogen receptor modulators; often the protective effects were not attributable to the therapeutic mechanism of action. The time scale of protection and multi-drug protection suggested to the authors that at least some of the protectants must be acting intracellularly rather than directly interacting with the toxin. Another interesting observation was that seven of the drugs protected against neomycin and gentamicin exposure but not kanamycin exposure, suggesting that kanamycin kills hair cells via a different mechanism. Two drugs, benzamil and paroxetine, protected from aminoglycoside- *and* cisplatin-induced hair cell loss. This result suggested that the aminoglycosides and cisplatin act at least in part to kill hair cells via similar mechanisms. By contrast, other studies in zebrafish suggest that the mechanisms must be different (Owens et al., 2008). In reality, it is probably a combination of the two.

### 1.7.5 Screen to identify compounds that improve regeneration following ototoxic insult

Studies in zebrafish have also diversified to include the assessment of compounds thought to aid hair cell regeneration as zebrafish, unlike humans, show regenerative capability after toxic insult. In 2003, it was shown that hair cell regeneration after treatment with neomycin could be seen between 12 and 24 hours post treatment and involved increased proliferation in support cells (Harris et al., 2003). Moreover, the observed regeneration was dose-dependent; neuromasts treated with a lower dose of neomycin were able to regenerate hair cells to near control levels whereas at higher doses, fewer hair cells were observed. This is also the case in larvae treated with copper (Hernandez et al., 2006).

A small number of potential enhancers of regeneration have been discovered through drug screening. For example, fucoidan, a purified natural compound found in seaweed, enabled enhanced regeneration 12 and 16 hours after neomycin treatment. Although fucoidan does not prevent cell death, it has been shown to stimulate supporting cells to proliferate and presumably give rise to the regeneration of associated hair cells (Moon et al., 2011). Just this year (2012), Dexamethasone and prednisolone have been identified as enhancers of regeneration following neomycin-induced toxicity. These compounds potentiated hair cell numbers by increasing mitotic activity (Namdaran et al., 2012).

### 1.7.6 Assessing the functional affects of ototoxins in the zebrafish

Compared to cell based histological assays there has been a relative paucity of research into the functional consequences of ototoxin-induced hair cell damage. At the start of my study, no functional screens had been performed to identify ototoxins.

In 2007, Johnson and colleagues reported that copper decreases the ability of larvae to orient to a current (rheotaxis). More recently two papers have been published that identify rheotaxis as a behaviour that could be harnessed to measure functional hair cell damage (Olszewski et al., 2012; Suli et al., 2012). These papers have only explored the effects of neomycin on rheotaxis, however.

In larvae and adults, the auditory evoked startle is an important behaviour, mediated by the lateral line. Startle is pivotal in predator evasion (see Chapter 4). Despite this, there are virtually no published data looking at the effect of ototoxin exposure on this important reflex. Just one study, using a flow stimulus to induce the startle response, has shown that neomycin treatment decreases responsiveness to water flow (McHenry et al., 2009). This is despite the fact that Zeddies and Fay highlighted the potential for the development of quick and robust assays of hair cell damage with a functional readout as long ago as 2005 (Zeddies and Fay, 2005). The lack of data on the functional effects of ototoxins highlights an area where more work can be done.

### 1.7.7 Available tools to study ototoxicity in the zebrafish lateral line

1.7.7.1 The use of vital dyes to study neuromasts

A range of vital dyes have frequently been used in vivo to visualize the neuromasts of the PLL in real time (Figure 1.7). In the study described in this thesis, hair cell damage is determined by the use of vital dyes. In previous studies, the dyes DASPEI, YO-PRO and FM1-43 have been used most frequently to visualise the hair cells of the PLL (e.g. Chiu et al., 2008; Harris et al., 2003; Seiler and Nicolson, 1999; Whitfield et al., 1996). See Chapter 3 for a more detailed description concerning the vital dyes used in this study. Typically, assays employ vital dye staining after compound exposure and then score the hair cell damage using arbitrary scoring system based on the degree of damage/fluorescence observed.

#### 1.7.7.2 The use of transgenic lines to study the lateral line

Over the last decade, a number of zebrafish transgenic lines have been created that enable the visualisation of the hair (Kindt et al., 2012; McDermott et al., 2010; Parinov et al., 2004; Xiao et al., 2005) and accessory cells of the neuromasts (Behra et al., 2009; Behra et al., 2012; Haas and Gilmour, 2006; Parinov et al., 2004), as well as their supplying nerves and glia (Faucherre et al., 2009; Gilmour et al., 2002) and that monitor the mechanotransductive capacity of the hair cells (Kindt et al., 2012). These transgenic animals are valuable tools not only in investigating lateral line development and hair cell regeneration, but potentially also in visualising ototoxin-induced damage (Figure 1.8).

For example, the *cldnB::GFP* line has been used to great effect to study the pathways affecting lateral line migration and neuromast deposition and to observe the migration of leukocytes to neuromasts during the inflammatory response in vivo (d'Alençon et al., 2010; Haas and Gilmour, 2006).

The *Tg(pou4f3::mGFP)s356t* (or *Brn3c::mGFP*) and *sqET4* lines mark hair cells of the lateral line and ear; experiments using these lines have shown the effects of ototoxins on both sensory systems (Behra et al., 2009; Buck et al., 2012; Choi et al., 2011; Hernandez et al., 2007; Moon et al., 2011; Olivari et al., 2008; Parinov et al., 2004; Xiao et al., 2005).

Most recently, the *Cameleon* transgenic line has been developed to study the early onset of mechanotransductive activity of hair cells that precedes the uptake of FM1-43. *Cameleon* utilises D3cpv, a genetically encoded FRET (fluorescence resonance energy transfer)-based calcium indicator to visualise mechanotransductive events specifically in hair cells. Using the line, it has been shown that the kinocilium is required for the onset of mechanotransduction in immature hair cells lacking mechanotransduction channels and tip links (Kindt et al., 2012).

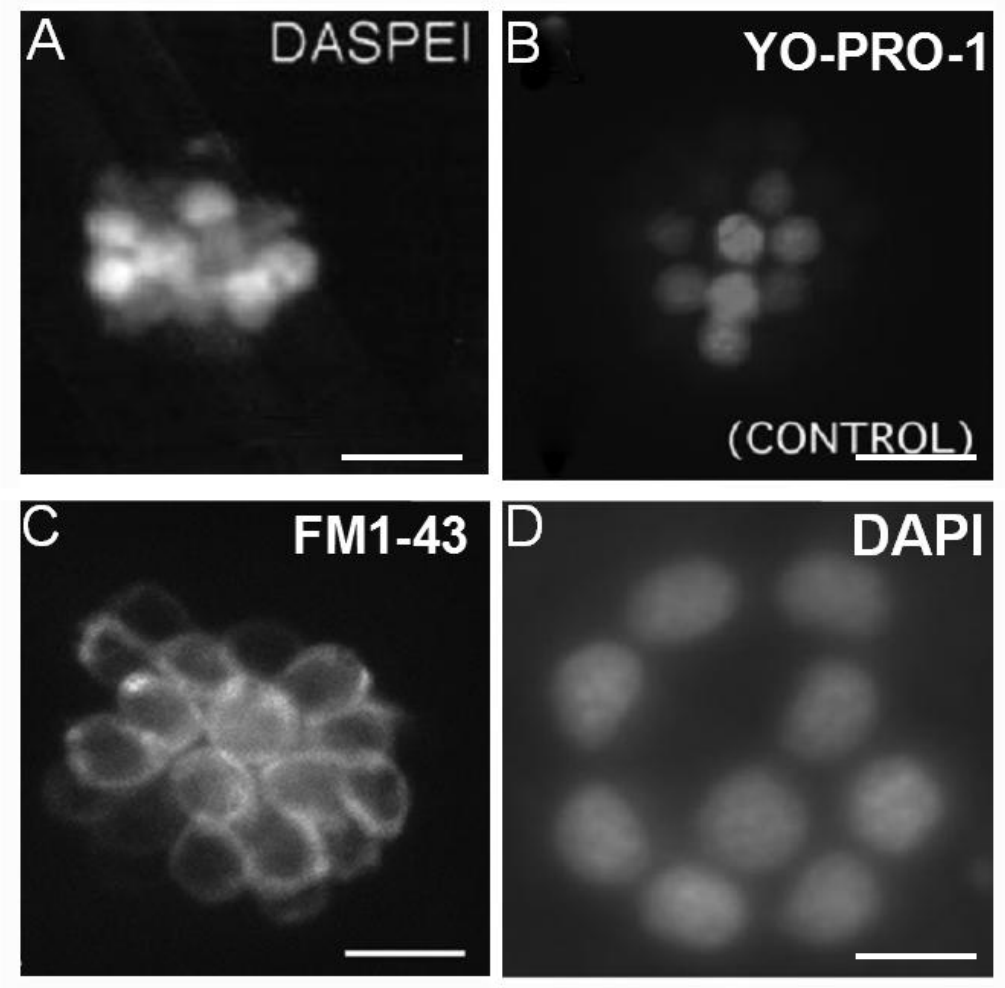



**Figure 1.7: Hair cell staining in the larval zebrafish.** A composite image of hair cell vital dyes. (A) The mitochondrial vital dye DASPEI stains for hair cells in the neuromast with 15 minute exposure time. Scale bar = 20 µM. (B) The dye Yo-Pro-1 stains the nuclei of the hair cells. Scale bar = 25 µM. (C) FM1-43 labels mechanotransductively active hair cells and is fixable in the 'FX' form. Scale bar = 10 µM. (D) Like YO-PRO-1, DAPI also stains the nuclei of the hair cells. Scale bar = 12.5 µM. Images for composite figure (A) Harris et al., 2003; (B) Chiu et al., 2008 and (C) Ou et al., 2007.

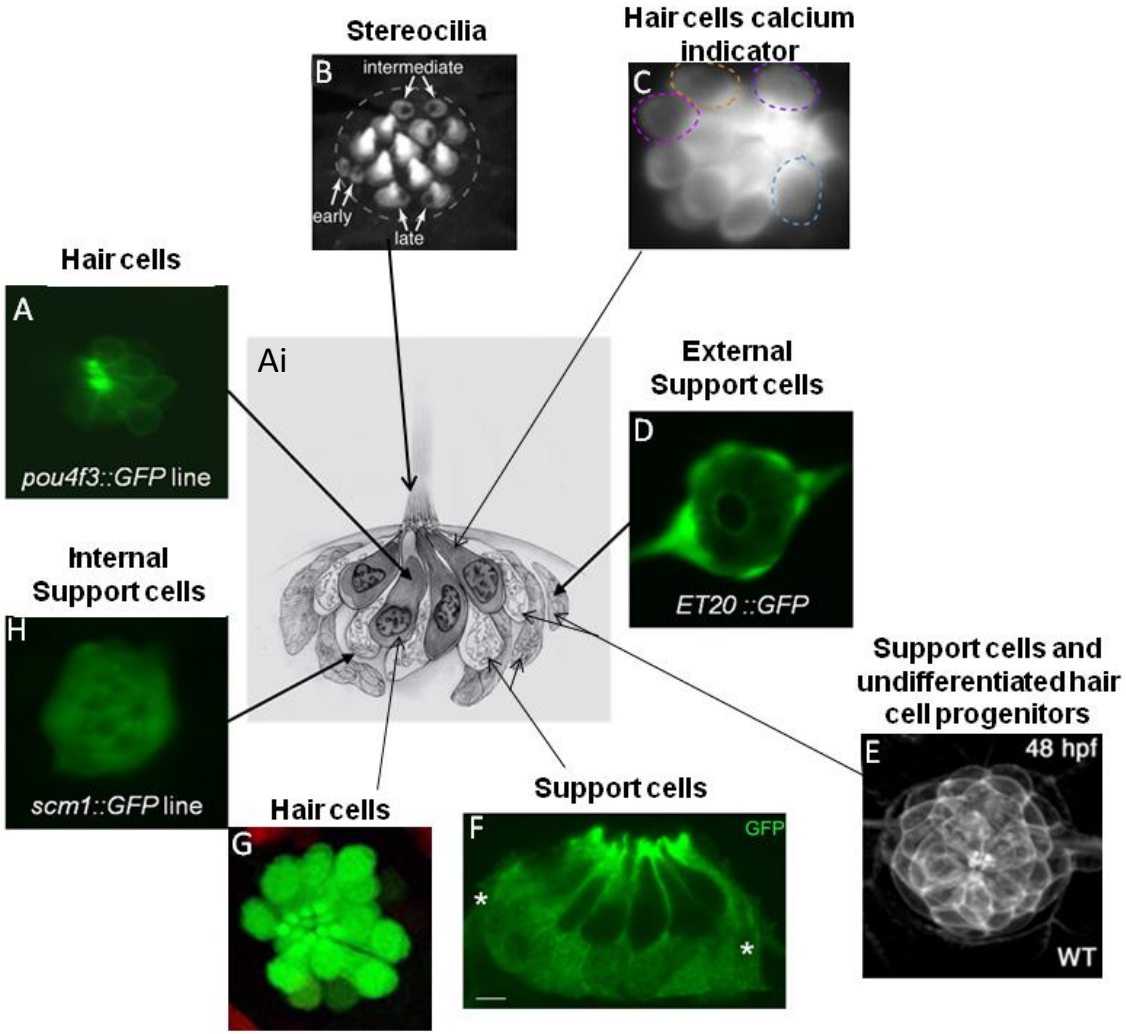



**Figure 1.8: Transgenic lines used to study neuromasts.** (Ai) A representative drawing of a lateral line neuromast. (A) The *pou4f3::GFP* line marks hair cells of the neuromast. (B) *Tg(myo6b:b-actin-GFP)* marks hair cell bundles. (C) The *cameleon* transgene acts as a calcium indicator in hair cells. (D) The enhancer trap *ET20::GFP* line marks the mantle cells. (E) *Tg(cldnb:lynGFP)* marks support cells and undifferentiated hair cell progenitors. (F) *Tg(tnks1bp1:EGFP)* marks all support cells. (G) The *ET4::GFP* line marks hair cells. (H) *scm1::GFP* marks the internal support cells. Images (Ai), (A), (D) and (H) taken from Moon et al., 2011. Images (B) and (C) taken from Kindt et al., 2012. Image (E) taken from Matsuda and Chitnis, 2010. (F) Taken from Behra et al., 2012. (G) Taken from Wibowo et al., 2011.

## 1.8 Project Aims

Despite the variety of research into the zebrafish as a disease model for ototoxicity, there is still a level of scepticism regarding their real value within the field of safety pharmacology (Redfern et al., 2008).

The overall aim of the current study was to investigate the potential value of the zebrafish as an alternative non-mammalian model of drug-induced ototoxicity. There were 3 main questions associated with this. Is the zebrafish auditory and vestibular system responsive to a range of known mammalian ototoxins in a concentration dependent manner; does any observed histological damage result in a reduction in auditory function; and is one or more of the mechanisms known to induce ototoxicity in mammals, comparable in zebrafish? (See Figure 1.9).

From this, the specific objectives of the project were to:

- Investigate the pathological consequences of exposure to a small collection of known human ototoxins, with differing mechanisms, on the zebrafish lateral line neuromasts and inner ear.

- Develop and optimise a semi-automated method to provoke and assess the naturally occurring noise-induced startle reflex in zebrafish.

- Exploit the above method (in combination with additional simple reflex-based assays) to investigate the functional consequences of exposure to the selected ototoxins.

- Probe the underlying mechanisms of ototoxin-induced damage in a single compound of interest, and compare the results obtained to what is reported in the literature from non-human model systems.

The results chapters that follow document the progress of the project in terms of the above aims. Chapter 3 describes the pathological consequences of exposure to ototoxins on both the lateral line and ear. Chapter 4 comprehensively details the development and optimisation of the startle assay. The methods used in Chapter 5 draw on results from Chapters 3 and 4 in order

to give an account of the functional consequences of ototoxin exposure. Chapter 6 and Appendix 3 report some preliminary data on the underlying mechanisms of cisplatin-induced damage in zebrafish and describe the potential future directions for the project.

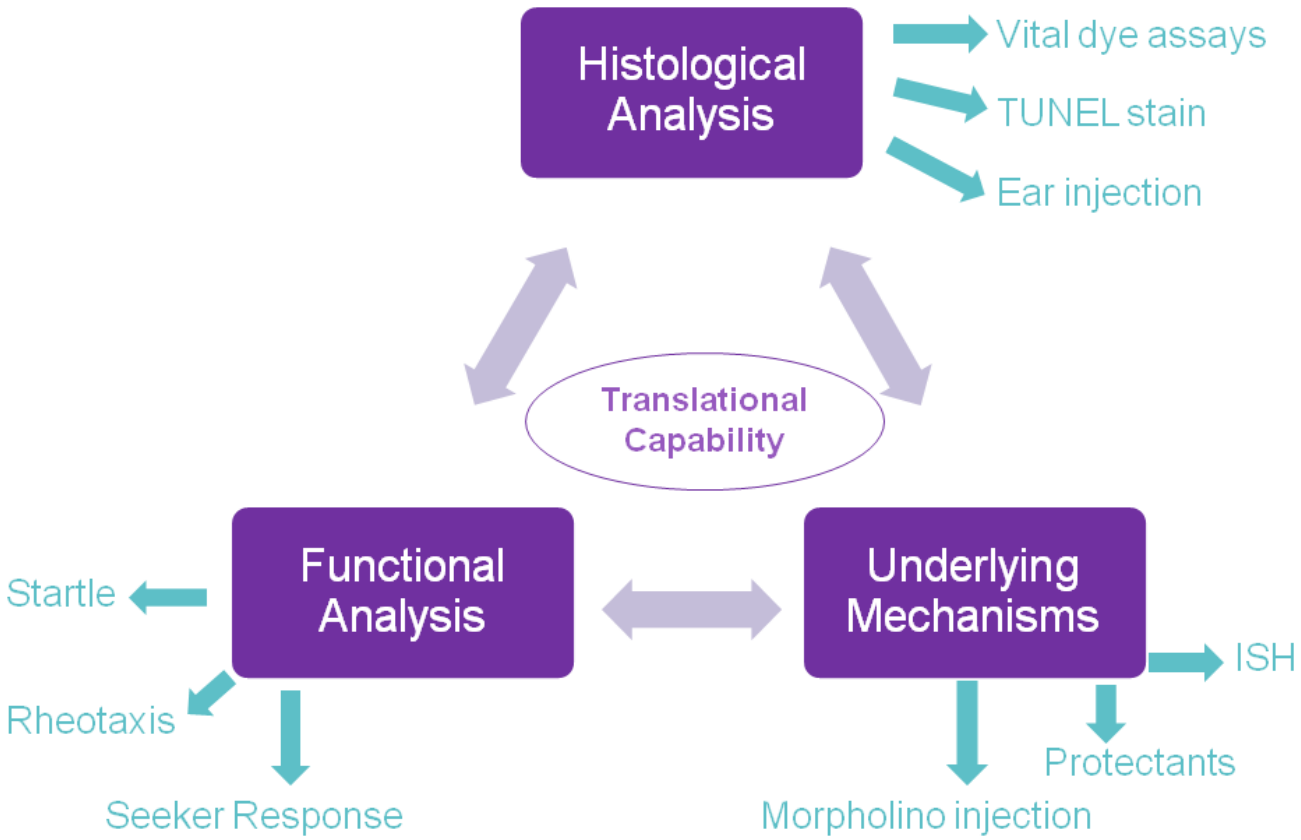



**Figure 1.9: Project aims.** Histological and functional analyses will act as indicators of ototoxicity. Histological investigations will centre around vital dye assays, TUNEL staining for cell death. Otic injections will act to assess the effects of potential ototoxins in the ear. Functional assays will test for the larval rheotactic response, auditory evoked startle response and underwater motion detection by larvae. Time permitting, experiments will be performed to assess the underlying mechanisms of damage caused by a single ototoxin.

# Chapter 2 Materials and Methods

## 2.1 Zebrafish husbandry

Wild-type zebrafish (AB strain, Sheffield brood stock, UK) adults were used both at Sheffield and Brixham Environmental Laboratory. *Tg(pou4f3::mGFP)s356t* and *i193* adult zebrafish (Xiao et al., 2005; Stone Elworthy (unpublished line)) were used at Sheffield only. Adult fish were maintained on a 14 hour light/10 hour dark cycle according to standard protocols (Nüsslein-Volhard and Dahm, 2002) and induced to breed using group spawning tanks. Egg collection and staging was performed according to standard procedures (Kimmel et al., 1995; Nüsslein-Volhard and Dahm, 2002). Larvae were raised at a density of 50 (or 128 in the case of the raising density experiments) per 90 mm Petri dish (Sterilin, Newport, UK), at 28°C (± 1.0°C) in standard E3 culture medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.0001% methylene blue). For vital dye staining, fish were transferred into E3 culture medium without methylene blue at 2 days post fertilisation (dpf) to prevent methylene blue uptake into cells. All experiments were carried out on larvae below 5.2 dpf.

To anaesthetise larvae, 1 mL of 4% MS222 (3-aminobenzoic acid ethyl ester; CAS No. 886-86-2; Sigma-Aldrich, UK) was used per 30 mL of E3 solution (400 mg Tricaine powder, 97.9 mL distilled water and 2.1 mL 1M Tris buffered to pH7).

## 2.2 Imaging

Anaesthetised larvae were mounted in 1% low melting point agarose (CAS No. 9012-36-6; Sigma-Aldrich, UK) and imaged using a BX51 compound microscope, Camedia (C-3030ZOOM) camera and Cell B software (Olympus, UK). Confocal images were acquired using an Olympus FV1000 confocal with SIM-scanner on a BX61 upright microscope. Images were compiled using Adobe Photoshop software (Adobe Inc., USA).

## 2.3 Choice of compounds

### 2.3.1 Ototoxins

All positive compounds were selected from literature searching, based on a reported ototoxic effect in humans and non-human mammals.

### 2.3.2 Negative control compounds

A small subset of negative control compounds, selected to have similar pharmacological effects in humans to the ototoxins, but no documented ototoxic side effects, were also tested. According to the literature these compounds exhibited no additional reported behavioural, locomotor or visual effects.

### 2.3.3 Identification of compounds for mechanistic investigations

A literature search was carried out to identify chemical compounds that were closely linked to the mechanisms thought be involved in cisplatin-induced hair cell death. Compounds were selected based on their activation/inhibition of various pathway components from cell and mammalian experimental studies.

## 2.4 Compound exposures

### 2.4.1 Suppliers

All compounds and reagents were obtained from Sigma-Aldrich (UK), unless otherwise stated.

### 2.4.2 Approximation of appropriate compound exposure concentrations

2.4.2.1 Literature searching

A literature search was used to identify appropriate dosing levels. When there were no zebrafish data available, values were approximated from cell line data, mammalian toxicity testing and/or Body Surface Area (BSA) calculations (see below).

2.4.2.2 BSA calculations

An attempt was made to estimate the concentration in human blood per treatment for certain compounds that lacked human plasma exposure data. Assuming the solution containing the compound of interest was in equilibrium with the lateral line hair cells, it was then possible to use these equivalent exposure concentrations (or higher) in order to focus the concentration range used for the initial MTC. This was done by:

Calculation of the BSA of the average English adult using the Mosteller formula:

$$BSA(m^2) = \sqrt{\frac{weight\ (kg) x\ height\ (cm)}{3600}}$$

Where average adult height* = 168.3 cm and average adult weight*[2] = 76.9 kg

Calculation of total dose based on recommended dose:

$$Total\ dose = BSA\ (m^2) x\ recommended\ dose\ (mg/m^2)$$

Calculation of the molar concentration of compound in 5L of blood based on total dose (mg):

$$Blood\ concentration\ (M) = \frac{(Total\ dose\ (mg) \div molecular\ weight)}{5000(mL)}$$

5 L blood is assumed to be the total blood volume in an average adult.

### 2.4.3 Initial exposures

Initial exposures were performed in 24-well plates (Corning Costar, NY, USA) by direct immersion of larvae (5 dpf) in a range of solutions across a defined concentration range. As a control, larvae were immersed in E3 alone (also referred to as dilution water control (DWC)) or E3 containing 0-2% DMSO or methanol as a solvent control (SC). The Maximum Tolerated Concentration (MTC) of each compound was determined (minimum of 6 larvae per concentration for each trial). The MTC was classed as the concentration of compound that induced ≥20% mortality after a set exposure time (for exposure times, see Table 2.1). If a compound failed to reach the MTC, a second trial was performed.

### 2.4.4 Adjusted exposures

Using the MTC data, appropriate concentration ranges and exposure times were established for the definitive compound exposures and subsequent hair cell damage, functional and mechanistic assessments. Treatment times and concentrations were specific to each compound tested (Table 2.1). For mechanistic studies, 2 hour co-treatments of cisplatin ($IC_{50}$/450 µM/900 µM) and the drug of interest at varying concentrations were performed. After compound exposure, larvae were rinsed 3 times with E3 and left to recover for 60 minutes prior to further processing. For each exposure, an appropriate DWC/SC was

* Data for the average English adult (> 16 years of age) were taken from "Health Survey for England 2008". http://www.ic.nhs.uk/. 17-12-2009.Retrieved 10-03-2012.

used alongside a positive control, which was neomycin (100 or 300 µM dependent upon assay type). Compound exposures were performed in an identical manner in *Tg(pou4f3::mGFP)s356t* larvae. For full details, see Table 2.1.

**Table 2.1: Test compound exposure details.** Treatments were performed as shown for each assay. MTC was defined as the concentration of compound to elicit more than 20% mortality within a single treatment group.

| Test compound | CAS No. | DASPEI assay | | Startle, SR and Rheotaxis Assays | | MTC (>20% mortality) (mM) |
|---|---|---|---|---|---|---|
| | | Concentration range (µM) | Exposure time (Hours) | Concentration range (µM) | Exposure time (Hours) | |
| Neomycin | 1405-10-3 | 0-300 | 1 | 0, 14, 100 | 1 | 0.5 |
| Streptomycin | 3810-74-0 | 0-300 | 1 | 0, 40, 200 | 1 | 4 |
| Gentamicin | 1405-41-0 | 0-1000 | 1 | 0, 25, 1000 | 1 | >10 |
| Cisplatin (batch 029K1426) | 15663-27-1 | 0-100 | 2 | 0, 14, 100 | 2 | 1 |
| Cisplatin 069K1236 and 479306 (Aldrich) | 15663-27-1 | 0-1000 | 2 | 0, 14, 100 | 2 | 1 |
| Aspirin | 50-78-2 | 0-400 | 1 | 0, 193, 300 | 1 | 1.5 |
| Furosemide | 54-31-9 | 0-1000 | 2 | - | - | variable |
| Copper (II) Sulphate | 7758-98-7 | 0-1 | 1 | 0, 0.5, 1 | 1 | 0.15 |
| Amoxicillin | 26787-78-0 | 0-2000 | 2 | 0, 1000, 2000 | 2 | 12 |
| Cefazolin | 27164-46-1 | 0-5000 | 1 | 0, 2500, 5000 | 1 | >20 |
| Melphalan | 148-82-3 | 0-400 | 0.5 | 0, 200, 400 | 1 | >0.5 |
| Gemcitabine | 122111-03-9 | 0-5000 | 0.5 | - | - | 1.5 |
| DPI | 4673-26-1 | 0-62.5 | 2 | - | - | >0.125 |

## 2.5 Vital dye staining of neuromasts

### 2.5.1 DASPEI

The fluorescent vital dye DASPEI (2-[4-(dimethylamino)styryl]-N-ethylpyridinium iodide; CAS No. 3785-01-1) was used to label the mitochondria of hair cells within the lateral line neuromasts (Balak et al., 1990). Following compound exposure and recovery, larvae were incubated in 2 mL E3 containing DASPEI (0.05 mg/mL) for 20 minutes under dark conditions. Subsequently, larvae were rinsed 3 times with E3.

### 2.5.2 FM1-43FX

FM1-43FX (Invitrogen; F-35355) was used as an indicator of the mechanotransductive activity of hair cells (Betz et al., 1996; Gale et al., 2001) within the lateral line. Following compound exposure and recovery, larvae were immersed in 2 mL 3 µM FM1-43FX for 45 seconds in the dark. Larvae were then rinsed 3 times with E3.

### 2.5.3 DAPI

DAPI (CAS No. 28718-90-3) was used as a live nuclear stain in the lateral line. Subsequent to compound exposure and recovery, larvae were immersed in 2 mL of 0.1 µg/mL DAPI solution for 15 minutes in the dark. Larvae were then rinsed 3 times with E3.

## 2.6 Optimisation of DASPEI staining

### 2.6.1 Duration of staining

Larvae were immersed in DASPEI (0.05 mg/mL) for 5, 10, 15, 20, 25 and 30 minutes and rinses performed. DASPEI scoring was performed to assess the most appropriate duration of exposure (i.e. the concentration that gave the strongest staining).

### 2.6.2 The effect of pH

2.6.2.1 Untreated control larvae

The effect of pH on DASPEI staining was measured in small numbers of larvae. Small glass beakers were filled with 20 mL of E3 solution (no Methylene blue). The pH of each beaker was adjusted manually with 1M hydrochloric acid (CAS No. 7647-01-0) and 1M sodium hydroxide solution (CAS No. 1310-73-2). Five larvae were added to each beaker in a further 5 mL of fluid and an initial pH

reading was taken. Larvae were exposed to the solution for 1 hour, followed by a final pH measurement. Larvae were then rinsed three times in E3 and recovered for a further 60 minutes. Staining and scoring was performed as described (Sections 2.5.1 and 2.7.1).

2.6.2.2 Compound treated larvae

The effect of pH on DASPEI staining during compound treatment was investigated. Treatments were carried out in 24 well plates. Non pH altered compound treatments and pH altered treatments were performed simultaneously, with corresponding water/solvent controls. For non-altered compound treatments, the treatment was as in Section 2.4.4, but with additional pH readings before and after the incubation period. For pH altered samples, the pH of a 2X working solution (10 mL total volume) was altered before further diluting to 1X with embryo solution containing the larvae (2 mL total volume in multi-well plates). The pH readings were taken in the well plate using a Corning pH meter 240 (Corning, USA) at the start of treatment to account for the addition of E3. A final pH reading was taken at the end of exposure to assess buffering capacity. Ten larvae were used for each treatment group. At the end of treatment, larvae were rinsed three times in E3 and DASPEI scored as in Section 2.7.1.

### 2.6.3 Effects of anaesthesia

Untreated, DASPEI-stained (Section 2.5.1) larvae were cooled on ice for 30 minutes; half were also treated with MS222 (Section 2.1). Scoring (Section 2.7.1) was performed to exclude the possibility that MS222 affected staining.

### 2.6.4 Testing automation of DASPEI scoring

Two systems were used to test the viability of an automated method of fluorescence measurement. Larvae were immersed in either DWC or 100 µM neomycin for 1 hour and washes performed as in Section 2.4.4. Following DASPEI staining and anaesthesia, larvae were transferred into a black-edged 96 well microplate. A total of two fish per well were aliquoted in a volume of 200 µL. Untreated animals were positioned in columns 1 to 6; treated larvae were placed in columns 7 to 12. A Phenosight high throughput high-content screening system was used to scan the plates at high speed. This was carried out with an exposure of 40 ms. Next, the plates were scanned at slower speed

with a 1 second exposure using a Nikon AZ100M microscope and NIS elements software (Version 3.00; see Figure 2.1). Multiple images were EDF processed using NIS elements software to create a single image per well. Thresholds were allocated to the images before conversion to binary using Image J (Version 1.45). Analysis of the mean gray value was carried out by defining a region of interest (ROI) along the pLL and taking two measurements per larva (ROI along the lateral line and comparison ROI along the body).

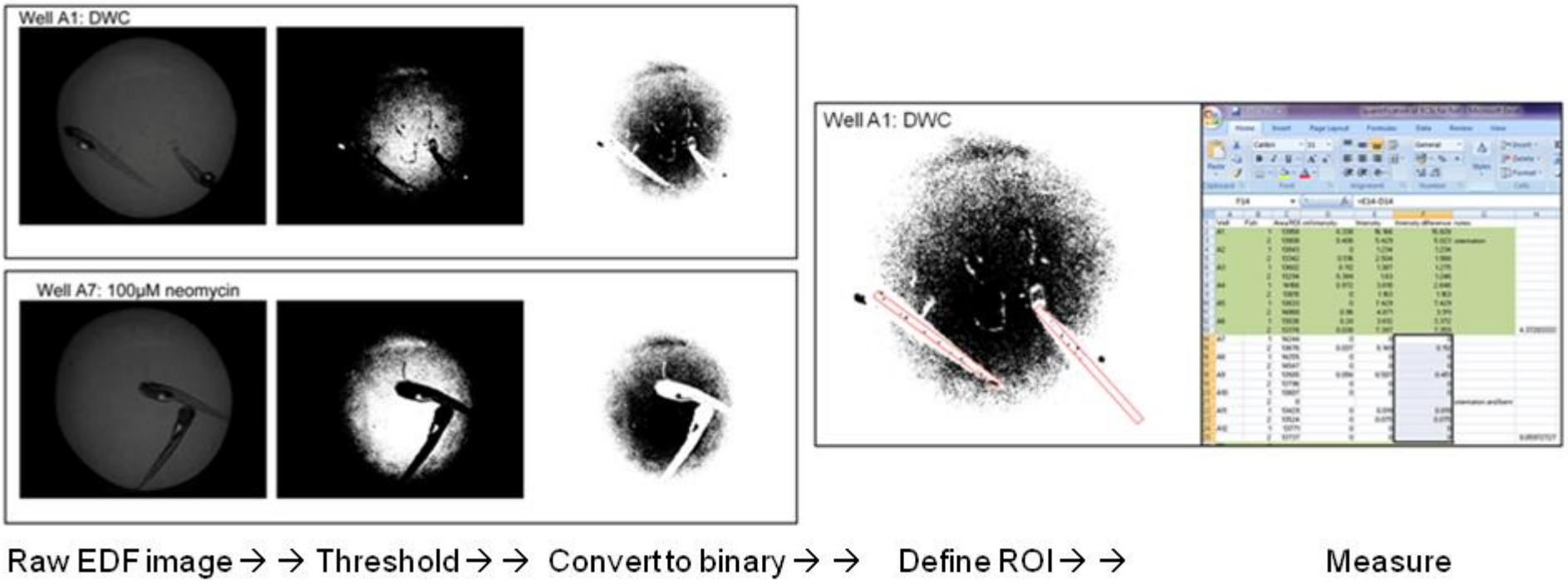



**Figure 2.1: Automated image processing of the DASPEI assay.** A Nikon AZ100M microscope and NIS elements software were used to capture the results of the DASPEI assay in control versus neomycin treated larvae (5 dpf). Image stacks were compressed into a single image using EDF processing. Single images were then thresholded and converted to binary using Image J. Regions of interest (ROI) were defined on each fish along the posterior lateral line. A mean gray value was taken for each animal.

## 2.7 DASPEI assay

### 2.7.1 DASPEI scoring technique

For scoring, larvae were anaesthetised in MS222 (as in Section 2.1) and observed under an epifluorescence dissecting microscope (Leica MZ-12 FLIII) equipped with a GFP1 filter set (excitation 425/60 nm; barrier filter 480 nm). Nine individual neuromasts of the posterior lateral line (pLL) that were present at 5 dpf were scored for fluorescence (Figure 2.2). The scoring scheme used was based on previous work (Harris et al., 2003) and was as follows: strong staining indicating the presence of hair cells, score = 2; weak reduced staining indicating the presence of fewer or damaged hair cells, score = 1; an absence of staining indicating the absence or severe damage of hair cells, score = 0. See Figure 2.2 for examples of neuromasts exhibiting strong and weak staining. Each fish was scored on both sides, giving a maximum total score of 36 per larva. A minimum of 8 larvae per treatment group per trial were scored and an average taken from 3-4 trials (except in the case of gemcitibine, where 5 larvae per treatment group were assessed over a single trial).

### 2.7.2 Methods employed to calculate $IC_{50}$ values

The concentration relating to an approximately 50% reduction in DASPEI score ($IC_{50}$) was ascertained for each compound using either log(concentration of test compound) vs. response (three parameters) or log(concentration of test compound) vs. response-variable slope (four parameters) curve-fitting equations (Prism 5.0 (GraphPad Software Inc., USA)). The best fit values (Section 3.15) were used later as the $IC_{50}$ values for the startle assay. Six positive and three negative compounds were selected for use in further behavioural assays to study the functional consequences of ototoxin exposure.

## 2.8 Ear injection technique

Anaesthetised *i193* larvae (4dpf) were mounted onto 38mm Petri dish lids in 1% low melting point agarose and imaged at 20x magnification with 2x zoom. The exposure time for each fish was noted. The otic lumen of the larvae was microinjected posterolaterally with ~4nL DWC, S/C or 'stock' solution dissolved in 10% phenol red. Larvae were left in situ for 30 minutes. Next, a syringe needle was used to free the larva from the agarose. The syringe needle was positioned under the tail of the larva so as not to cause damage and then gentle upward motion was used to release the animal. The head was released last. Larvae were then recovered individually in anaesthetic-free embryo medium for 4 hours at 28ºC. After viability and "tap" testing (tapping the Petri dish for an escape response), larvae were anaesthetised, remounted in the original orientation and imaged using the corresponding exposure time to assess damage (Figure 2.3).

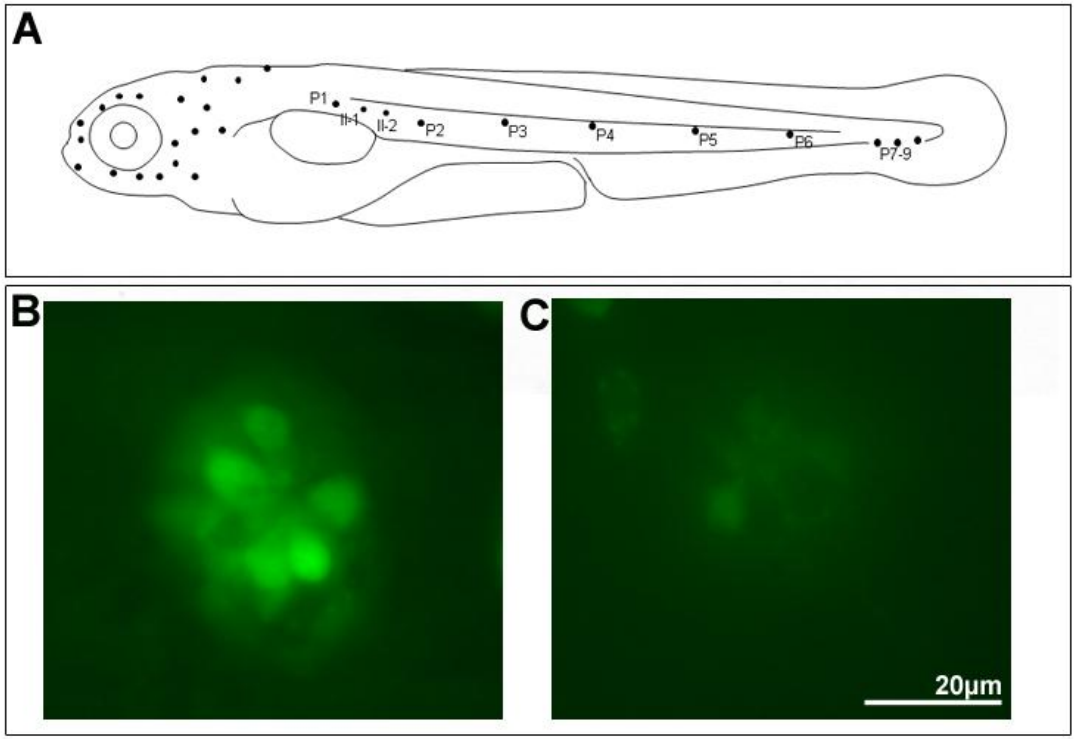



**Figure 2.2: Examples of DASPEI staining in pLL neuromasts.** (A) Schematic showing typical pattern of neuromasts in a 5 dpf zebrafish larva. Smaller neuromasts from the second wave of deposition are indicated (II-1, II-2), but were not scored for the purpose of the DASPEI assay. The remaining 9 neuromasts of the pLL (P1-9) were scored on both sides. (B) and (C) Exemplar images of neuromast staining with DASPEI. Image (B) is representative of a healthy neuromast given a score of 2 in the fluorescence assay. Image (C) is representative of a damaged neuromast, given a score of 1. Scale bar 20 µm.

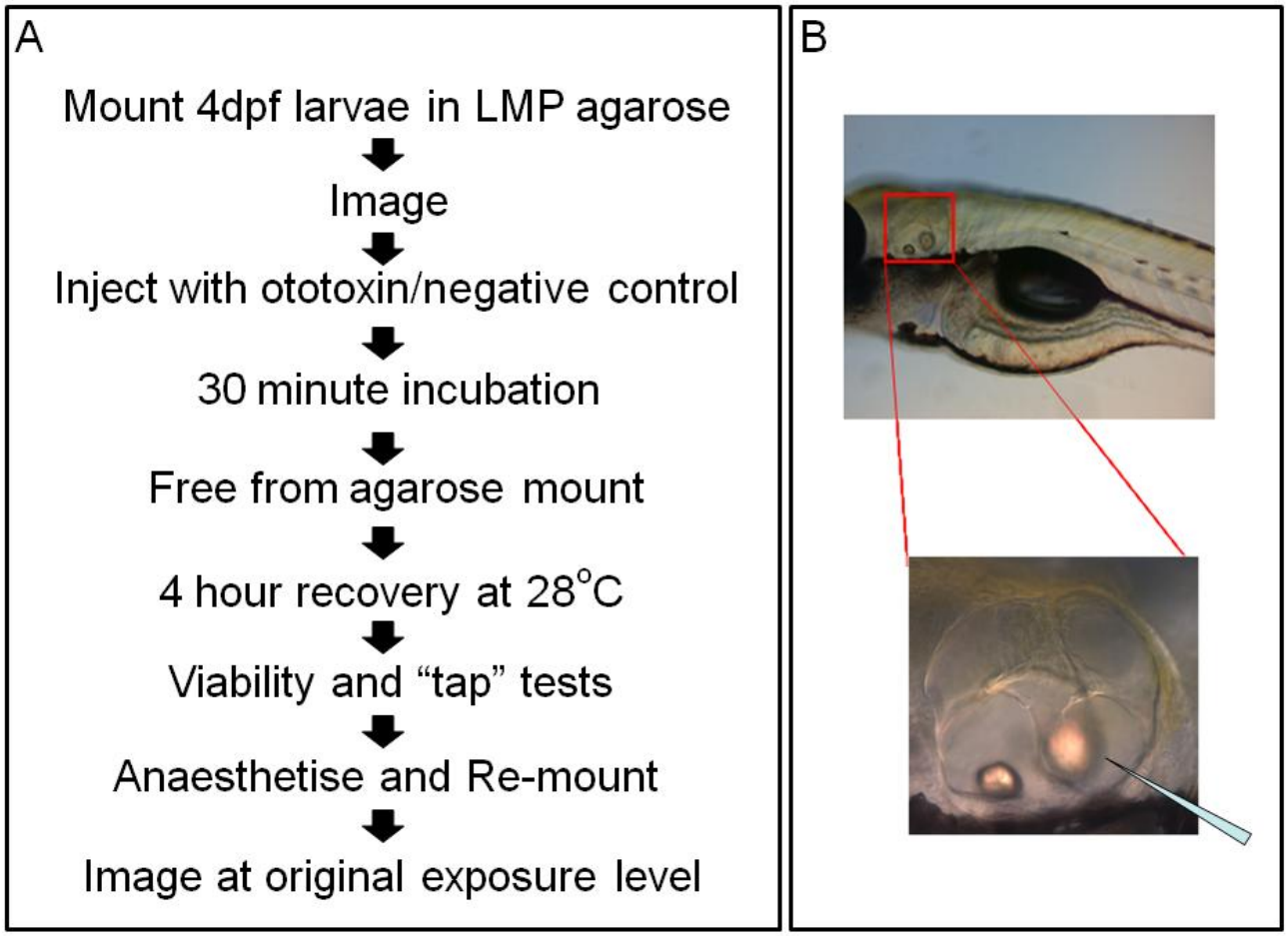



**Figure 2.3: Ear injection technique.** (A) The treatment and testing paradigm used to inject ototoxins. (B) Position of injection. Larvae were injected postero-laterally to avoid damage to the important structures including the otoliths and sensory patches.

## 2.9 Startle Assay

### 2.9.1 Equipment

2.9.1.1 Equipment list

- 15” (381 mm) 350W bass speaker range 35 Hz to 4 KHz (Maplin electronics, UK)
- 2.0 MHz PCGU1000 PC USB function generator (Velleman Inc., USA)
- SP103 amplifier (Acoustic Solutions, UK)
- 35 MHz 4-channel digital storage oscilloscope PM3305 (U) (Phillips, Netherlands)
- 0.3 megapixel high-speed digital video camera GRAS-03K2M (Point Grey Research Inc., Canada)
- TCM110 omni-directional electret-condenser microphone (RS Components Ltd., UK)
- Castle GA213 sound pressure level meter (Castle Group Ltd., UK)

2.9.1.2 Setup

A schematic of the experimental setup used to assess lateral line functionality is shown in Figure 2.4. Vibratory stimuli (sinusoidal/square tone bursts) were generated using a 2.0 MHz PCGU1000 PC function generator and controlled using PClab2000SE generator interface software (Velleman Inc., USA). Stimuli were amplified (SP103 amplifier; Acoustic Solutions, UK) and directed through a vertically oriented 15” (381 mm) speaker cone, housed in a wooden casing. Acoustic stimuli were delivered to the larvae through a 6.4 mm thick translucent plastic base plate, bolted to the speaker case, to which the microplate containing the larvae was fixed. Larvae under investigation were placed into 24-well plates with a single larva per well. The 24-well plate was securely bolted to the plastic base plate. Larval startle responses (auditory-evoked high speed movements, or AERs) were captured using a high speed 0.3 megapixel digital video camera (GRAS-03K2M, Point Grey Research Inc., Canada) set at a 60 frames/second capture rate. Recordings were visualised and processed using VideoTrack for Zebrafish™ software (Viewpoint Inc., France).

2.9.1.3 Calibration of the oscilloscope

All wave output parameters were measured using a calibrated 35 MHz 4-channel digital storage oscilloscope (PM3305 (U), Phillips, Netherlands). This was calibrated according to the manufacturer's instructions using calibration probes (see Figure 2.5).

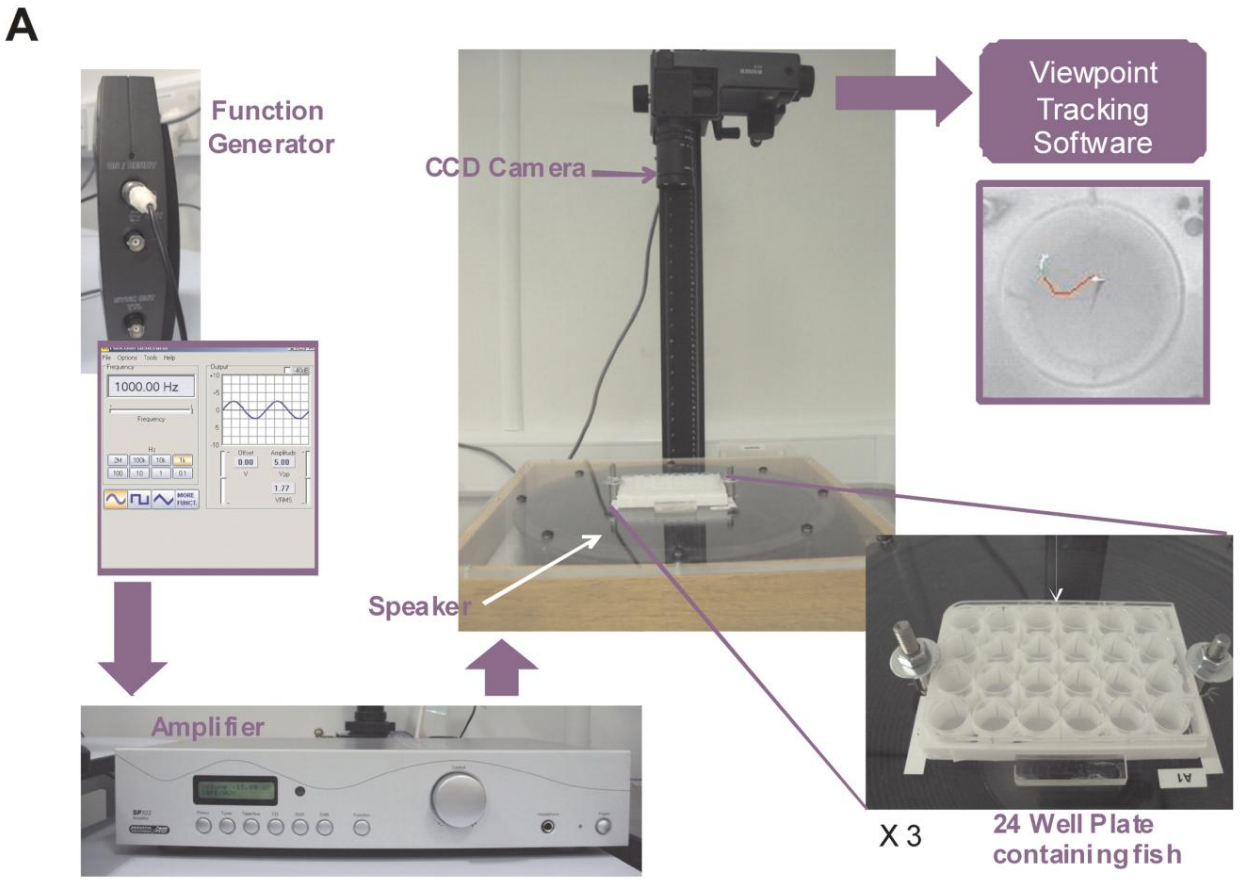
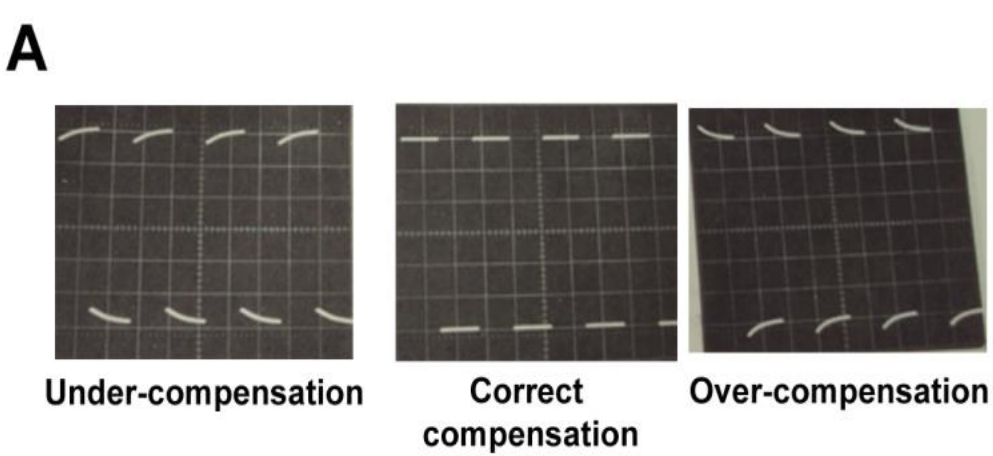



**Figure 2.4: Experimental setup used to evoke startle responses in larval zebrafish.**



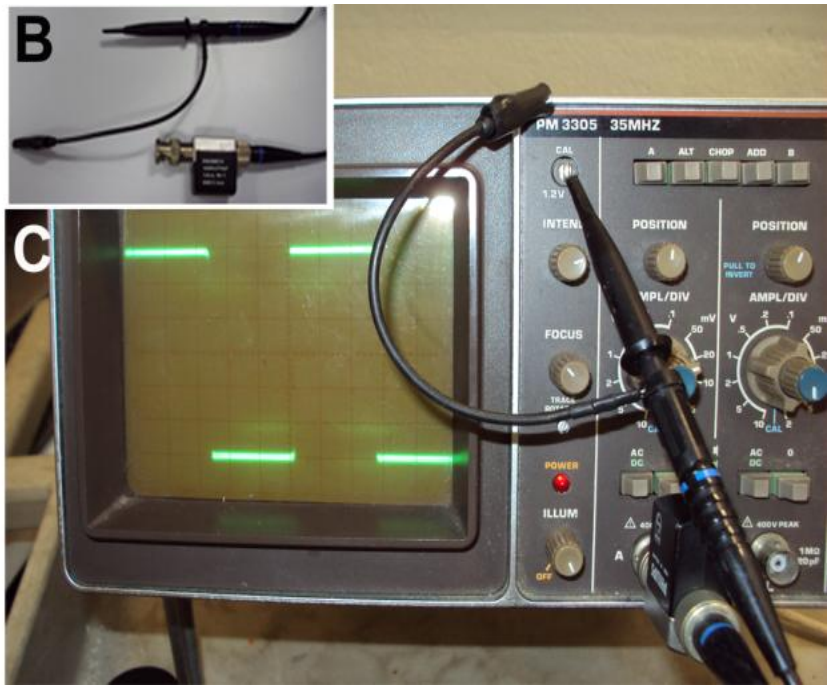




**Figure 2.5: Calibration of the oscilloscope.** The oscilloscope was adjusted using a trimmer and measuring pin (B), which was attached to oscilloscope (C). Adjustments were made to ensure correct compensation (A).

#### 2.9.1.4 Calibration of function generator for voltage and frequency

The function generator was calibrated using the oscilloscope to ensure that it had the correct output at each test frequency. Continual sinusoidal pure tones were generated and the output observed on screen (Figure 2.6).

$$Oscilloscope\ Wave\ Period\ (ms) = X\ horizontal\ divisions\ per\ peak\ x\ time\ per\ division(ms)$$

$$Wave\ Frequency\ (Hz) = \frac{1000ms}{wave\ period\ (ms)}$$

$$Oscilloscope\ Amplitude\ (Vpp) = X\ vertical\ divisions\ peak\ to\ peak\ x\ voltage\ per\ division\ (V)$$

2.9.1.5 Calibration of function generator for stimulus duration

The PCGU1000 PC USB function generator software gave an inaccurate readout of stimulus duration. To assess the *true* duration of the stimuli that the larvae would experience, the function generator was calibrated to the oscilloscope and measured DC output using the 'memory' function. The discrepancy and true duration was determined as below:

$$DC\ output\ duration\ (ms) = X\ horizontal\ divisions\ of\ signal\ output\ x\ time\ per\ division\ (ms)$$

$$Output\ discrepancy(ms) = Duration\ of\ stimulus\ displayed\ (ms) - DC\ output\ duration\ (ms)$$

2.9.1.6 Sound pressure level measurement and calibration

The 17 mm diameter wells of a 24-well plate were too small for direct measurement of the sound pressure level (SPL) using a standard hydrophone. Therefore, to measure the SPL inside the wells of the microplate (indirectly), a small microphone (TCM110 omni-directional electret-condenser, RS Components Ltd., UK) was used. The microphone was waterproofed with a latex sheath and calibrated against a pre-calibrated SPL meter (Castle GA213; Castle Group Ltd., UK) in air, and subsequently in wells filled with 2 mL of E3 solution. Measurements were taken over all test frequencies at previously optimised voltage settings (1.6 to 4.5 Vpp) for a small range of amplifier volumes (-40 to -10 dBTP). The output of the microphone was routed through the calibrated oscilloscope providing a direct measure between the SPL meter and the microphone, and consequently the attenuation in air versus that in fluid. The SPL meter was clamped at 35 mm above one of the four central wells (well 4C). In addition, the microphone wire was adhered directly to the SPL meter, to ensure that it was vertically positioned and adjacent to the meter. The microphone recordings in either air or water were then taken inside the centre of the well adjacent to well 4C, 5 mm above the bottom of the well.

2.9.1.7 Acetone coating and leaching of plates

The external surface of clear polystyrene multi-well plates (Corning, UK) were frosted using acetone immersion to prevent visual startle cues from animals in neighbouring wells. This also served to improve image contrast for video recording. Multiple 2mm holes were drilled into the plastic mould and plates were then held in acetone (CAS No. 67-64-1) for 2 minutes (being careful not to

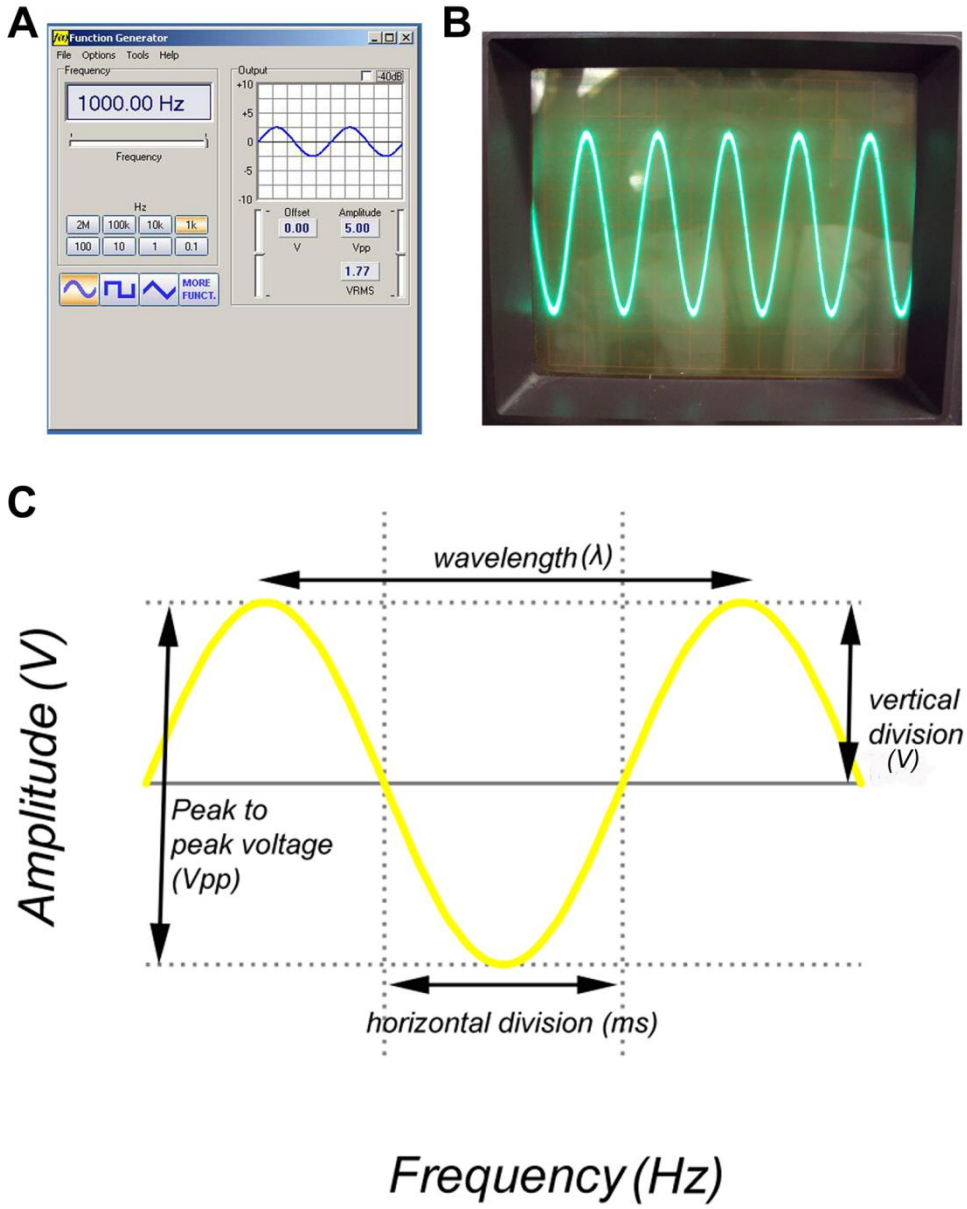



**Figure 2.6: Calibration of the function generator for voltage and frequency.** The output of the function generator was measured against the output displayed on the 4-channel oscilloscope (B) to calibrate the generator software (A). (C) Schematic of a pure tone sinusoid as viewed using an oscilloscope (with measurement parameters overlaid).

get any inside the wells). The plates were then immediately dipped in distilled water to rinse and left to air dry. Microplates (coated and non-coated) were leached of contaminants in running water overnight.

2.9.1.8 Testing stimulus spread in plates (salt test)

Dry sodium chloride (CAS No. 7647-14-5) was dispensed into each individual well of a microplate. A black card background allowed for video recording of the salt particles. A 20 second video recording was made with no stimulus, followed by a 200 Hz, -30 dBTP, 2.2 Vpp sine wave, finishing with a 200 Hz, -25 dBTP, 2.2 Vpp sine wave (Supplementary Material disk, “salt test”).

### 2.9.2 Stimulus form

2.9.2.1 Determination of optimum volume and voltage at individual frequencies using a custom hydrophone

Conditions were optimised for voltage peak to peak (Vpp) and amplifier volume (-dBTP) to ensure the resultant sound wave was not distorted across a range of frequencies (40-1200 Hz). Sinusoidal tones were generated for each frequency and were amplified onto the speaker. A hydrophone in a large beaker of water was attached to the speaker and connected to the oscilloscope to measure output. The maximum volume before distortion was ascertained at a range of voltages. Once the maximum non-distorted output was established, the response thresholds of control animals could be examined (Section 2.9.2.2). Following testing in the larvae, the maximum volume before non-distortion was re-checked using this same method.

2.9.2.2 Determination of response thresholds in control animals

Threshold responses of larvae were determined for each frequency to ensure that maximum larval responses occurred at the lowest possible volume and with non-distortion of the sound wave. A sub-threshold stimulus was given, which represented the lowest volume, and this was followed by 5 equally spaced sequential stimuli, up to a maximum volume threshold (the maximum volume attainable before distortion of waveform). Stimulus volume was chosen based on the best response at the lowest volume for each frequency. The responses of 24 larvae were tested over 3 repeated stimuli for each individual frequency.

2.9.2.3 Optimised stimuli

Stimuli consisted of sinusoidal or square waves of either 440 or 540 ms duration. The stimulus frequencies used were 40, 50, 100, 150, 200, 300, 400, and 500 Hz. See Table 2.2

**Table 2.2:** Optimised settings at each frequency.

| Frequency (Hz) | Duration (ms) | Voltage (Vpp) | Volume (dBTP) |
|---|---|---|---|
| 40 | 540 | 2.3 | -15 |
| 50 | 440 | 1.6 | -15 |
| 100 | 440 | 2.1 | -25 |
| 150 | 440 | 2.7 | -35 |
| 200 | 440 | 2.2 | -30 |
| 300 | 440 | 3.8 | -40 |
| 400 | 440 | 4.3 | -41.25 |
| 500 | 440 | 4.5 | -30 |

### 2.9.3 Scoring of the startle response

2.9.3.1 Scoring of startle responses by eye

Virtualdub freeware (version 1.9.11; http://virtualdub.org) was used to slow 60 frame/sec video recordings of control larvae to a fraction of their speed for analysis by eye. Startle responses were classed based on characteristics defined by (Burgess and Granato, 2007; Kimmel et al., 1974). Scoring was as shown in Table 2.3.

**Table 2.3:** Scoring larval responses by eye.

| Movement description | Classification |
|---|---|
| Larva is unresponsive to stimulus. Larva may move at other points during the recording. | U |
| Larva performs a movement in response to stimulus that has no large C- or S- bend. Not a classic startle movement. | M |
| Larva performs a timely but 'borderline' C-bend on stimulation (very mild startle) but not a strong C- or S-bend. | VMS |
| Larva performs a timely C-bend on stimulation (mild startle) but not a strong S-bend. | MS |
| Stereotypical S-bend followed by additional counter-flexion. Strong reaction. | S |
| Stereotypical S-bend followed by additional counter-flexion. Strong reaction at the edge of the well. | SE |
| Stereotypical S-bend followed by additional counter-flexion. Strong but slightly delayed reaction. | LS |

2.9.3.2 Setting a threshold for automated quantification of startle

The results of manual scoring from 20 individual videos of control 5 dpf larvae were played through the Viewpoint system and high-speed thresholds were adjusted to find the best threshold for startle. The three high-speed thresholds evaluated were 15.6 mm $s^{-1}$, 21.1 mm $s^{-1}$ and 26.6 mm $s^{-1}$. Assessments were made using Viewpoint colour traces. Two average values were measured to indicate the level of concurrence between score by eye and Viewpoint colour traces; one overall value that takes into account all animals (including non-responders; OPM) and one value that only takes into account traces for movement and startle (excluding non-responders; MSO). Thresholds were matched to the video clips so that larvae scored by eye as "S" (stereotypical startle), "SE" (startle at the edge of the well) and the majority scored as "MS" (a mild startle) could be visualised in the high-speed red threshold. Movements with no startle "M" (movement, no startle) were set as white or green (low/mid-speed) coloured and unresponsive animals ("U") were allocated to white (low-speed/no movement).

2.9.3.3 Comparison of scoring by eye versus automation

Twenty video recordings were compared in order to assess the concordance between scoring by eye and scoring by automated tracking at the high-speed threshold of >21.1 mm $s^{-1}$. The 20 files were scored by eye as in Section 2.9.3.1. The first set of comparisons made was for all measurements of movement (unresponsive/movement/startle). The second comparison was for the detection of *startle only*. Results were entered into an excel spreadsheet. When the manual and automated score matched, the cell on the excel spreadsheet was marked with a "1". When there was a discrepancy, the cell was left blank. A percentage match was calculated for each plate, and an overall percentage concordance was determined for each of the two comparison types.

### 2.9.4 Improving control compliance

2.9.4.1 Plate type

To test whether the capacity of the assay could be increased by using more wells per experiment, larval AERs were tested in either 24- or 48-well plates. For each individual test, larvae were equilibrated on the startle platform for a minimum of 30 minutes. Animals were then presented with eight selected frequencies (40, 50, 100, 150, 200, 300, 400 and 500 Hz) at the previously optimised voltages and volumes, with a duration of 440 ms or 540 ms. The ISI was 60-80 seconds. For both conditions (24- and 48-well plates), tests were carried out in triplicate with a total of either 24 or 48 larvae per trial. All subsequent experiments described used the 24-well plate format.

2.9.4.2 Wave type

One larva (5 dpf) was individually loaded into each well of a 24-well microplate and allowed to equilibrate for 30 minutes. Three stimuli (200Hz, 440 ms duration) of either a square or sinusoidal waveform were presented with a minimum inter-stimulus interval (ISI) of 60 seconds. The averaged AERs of the larvae for each wave type were then compared. All subsequent experiments described used the sinusoidal waveform.

#### 2.9.4.3 Density of raising

To assess the effect of the density of culture on animal startle responses, larvae were raised from 0-5 dpf at a density of 50 or 128 per Petri dish. Larvae were individually loaded into microplates, and left to equilibrate on the apparatus, as previously described (Section 2.9.4.2). Larvae were presented with 10 identical sinusoidal stimuli of 200 Hz (440 ms duration, 60-80 second ISI). This trial was repeated 3 times for each set of animals (24 larvae per test condition, per trial). To compare the response of animals raised at 50 or 128 per Petri dish, an average large distance (ALD) measurement (distance travelled at >21.1 mm $s^{-1}$) was taken for each animal over the 10 stimuli, and the ALD for all animals across 3 trials for each condition were calculated. To assess the development of fish housed at the two different densities, larvae were raised as described (Section 2.1) and imaged (for detail see Section 2.2).

#### 2.9.4.4 Choice of an appropriate Inter-stimulus Interval (ISI)

To determine possible habituation to acoustic stimulation, the response of larvae to varying ISIs was investigated. For each experimental trial, five separate ISIs (15, 30, 45, 60 and 120 seconds) were tested in turn on the same twenty-four test animals. Based on previous studies, a minimum gap of 15 minutes was placed in between each interval tested to allow animals to recover from any habituation (Best et al., 2008). For each ISI tested, multiple sinusoidal stimuli of 200 Hz were presented to larvae. The habituation experiment was carried out 3 times using different clutches of larvae. A test was also performed with a 1 second ISI, to exemplify true habituation.

### 2.9.5 Plate randomisation technique

An in-house program (PCRAN Version 1.01, AstraZeneca) was used to randomise the order of column treatment for each of the three plates in the final startle assay. A description of the treatment group was attached to the numbers one to six and the numbers allocated an order at random as detailed below (also see Appendix 2).

Number 1 = solvent control to discard (SC1)

Number 2 = solvent control to discard (SC2)

Number 3 = solvent control to use for statistical analysis (SC3)

Number 4 = $IC_{50}$ hair cell (as estimated by DASPEI assay)

Number 5 = MTC hair cell (as estimated by DASPEI assay)

Number 6 = Positive control (100 µM neomycin)

Test plate 1 (order from left to right in columns): 6 2 5 4 1 3

Test plate 2 (order from left to right in columns): 4 1 5 6 2 3

Test plate 3 (order from left to right in columns): 4 6 3 2 5 1

### 2.9.6 Final startle assay: Assessment of the larval startle response after ototoxin exposure

Larvae were individually loaded into each well in a total of 2 mL E3 medium, and left overnight. Three microplates were prepared per assessment, which provided data from 12 animals for each of the treatment groups. Before treatment, rheotaxis and seeker response testing was performed (Section 2.10). Larvae were then immersed in compound (see Table 2.1), and at the end of treatment, animals were rinsed 3 times in E3, and rheotaxis and seeker response scoring repeated. Larvae were then transferred to frosted microplates and placed onto the startle platform to equilibrate. A baseline recording of 60 seconds was taken at the beginning of equilibration to monitor pre-stimulation movement. Next, 8 sinusoidal tone bursts (Table 2.2) were presented with a randomised ISI of 60-80 seconds. During each stimulus, a 10 second video recording was taken to capture AERs. Video recordings were visualised and processed using VideoTrack for Zebrafish™ software (Viewpoint Inc., France) and Microsoft Excel. Each compound trial was carried out over 3 plates. For each plate, 3 control columns were assigned to ensure that a startle response could be identified against a baseline recording.

A single pre-determined control column per plate was used for statistical analysis to make animal numbers equal for each treatment group. To ensure

identical exposure conditions for all plates, treatments were staggered. Each compound was tested over 3 separate trials using different clutches of larvae, all at a temperature of 28 ± 0.5 °C (For full protocol proforma, see Appendix 2; Figure 2.8).

### 2.9.7 Data processing and macros for the startle assay

All datasets were processed using Microsoft Excel. For the final startle assay, a large amount of data was produced for each compound. To process this data efficiently, macros were created using the Excel macro facility (Appendix 1; also see Supplementary Material CD for files containing macros).

## 2.10 Measurement of rheotaxis and seeker response

Two additional assays were employed to investigate functional consequences of ototoxin exposure further: rheotaxis and seeker response. The same animals as in Section 2.9.6 were subjected to these tests.

### 2.10.1 Rheotaxis

For rheotaxis testing, 0.5 mL E3 was dispensed at high speed through a pipette to the side of individual wells in turn, resulting in a circular flow of fluid around the well. This was repeated, so that each larva was tested twice. Rheotaxis was scored as follows: 2 = immediate rheotaxis (orientation of the larva towards the direction of flow); 1 = rheotaxis observed on second attempt; 0 = no rheotaxis (See Figure 2.7).

### 2.10.2 Seeker response

To test the underwater motion detection and avoidance behaviour of larvae, seeker response (SR) was tested. For SR evaluation, a previously described method was used (Winter et al., 2008). Briefly, larvae were tested by gently approaching them from behind with a 20 µL pipette tip (the “seeker”). The response was scored as follows: 3 = uncompromised escape (immediate movement as soon as the tip touched the water surface, at a distance from the larva); 2 = reduced escape, which required the seeker to be moved towards the larva; 1 = escape following touch; and 0 = absence of an escape with touch (See Figure 2.7)

## 2.11 Measurement of baseline activity

To examine possible sedation effects of test substances, activity profiles were analysed from baseline recordings. Video footage of 60 seconds duration was taken for each test plate at the start of the equilibration time and the Viewpoint software was used to determine the average speed of each animal. Manual observation was also carried out throughout the 30 minutes. For comparison with animals sedated with anaesthetic, larvae were immersed in 0.1 µg/mL (full sedation), or 0.0125 µg/mL (light sedation), MS222 for 30 minutes. For the full startle assay and anaesthetic testing, a total of 72 larvae per trial were examined. Each compound was tested over 3 trials using different clutches of larvae.

## 2.12 Circadian rhythm

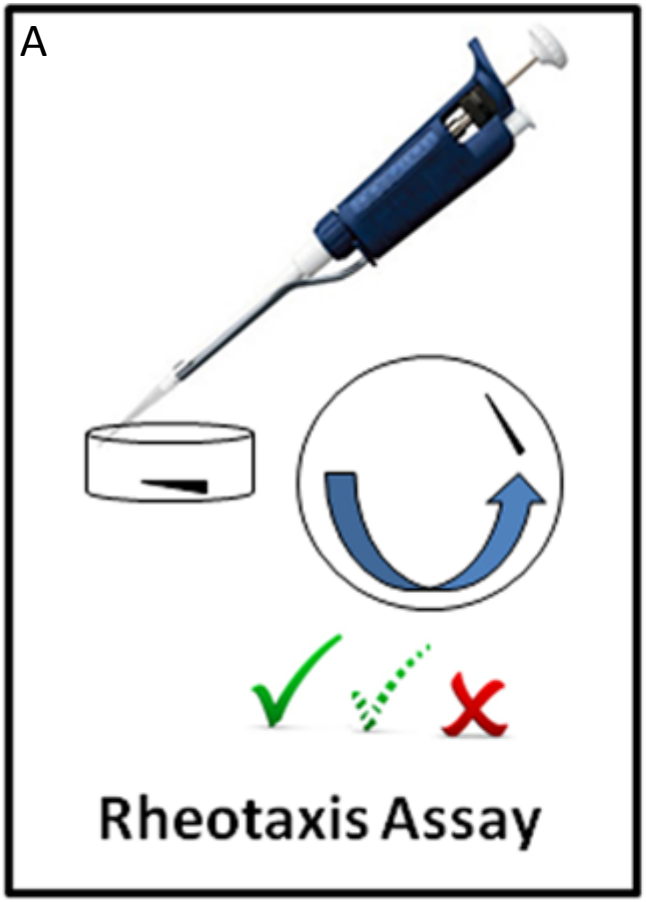
In light of evidence that circadian rhythm could affect movement, it was necessary to examine the effect of time of day on baseline activity and startle reactions. All animals for these experiments were kept in a temperature controlled room that was on an artificial light/dark cycle (14 hours of light/10 hours of dark) to stimulate circadian rhythm. The data for this analysis came from control animals taken on multiple days. The same clutches were compared for morning versus afternoon). Data pooled for all controls together were a comparison of 144 animals in each group. Data pooled for individual control columns were a comparison of 48 animals in each group.

## 2.13 Testing for AER threshold shifts with neomycin treatment

The usefulness of the startle assay in measuring threshold changes in the larvae was investigated following neomycin exposure. The same protocol as for the original startle assay was used (Appendix 2). The only changes made were in the treatment and the stimulation of larvae. Larval treatment groups were control (E3 medium) or 100 µM neomycin only, with three columns in each plate to achieve an n of 12 per treatment group. Stimulation of larvae was at 200 Hz frequency only but with a volume range of -30dBTP to -15dBTP (2.2 Vpp output). Data were pooled from a total of three trials on the same day.

## 2.14 Fixation and storage of embryos

Larvae were fixed in 4% PFA (Sigma-Aldrich, UK) at 4ºC overnight in a 1.5 mL microcentrifuge tube. For embryos younger than 2 dpf, embryos were



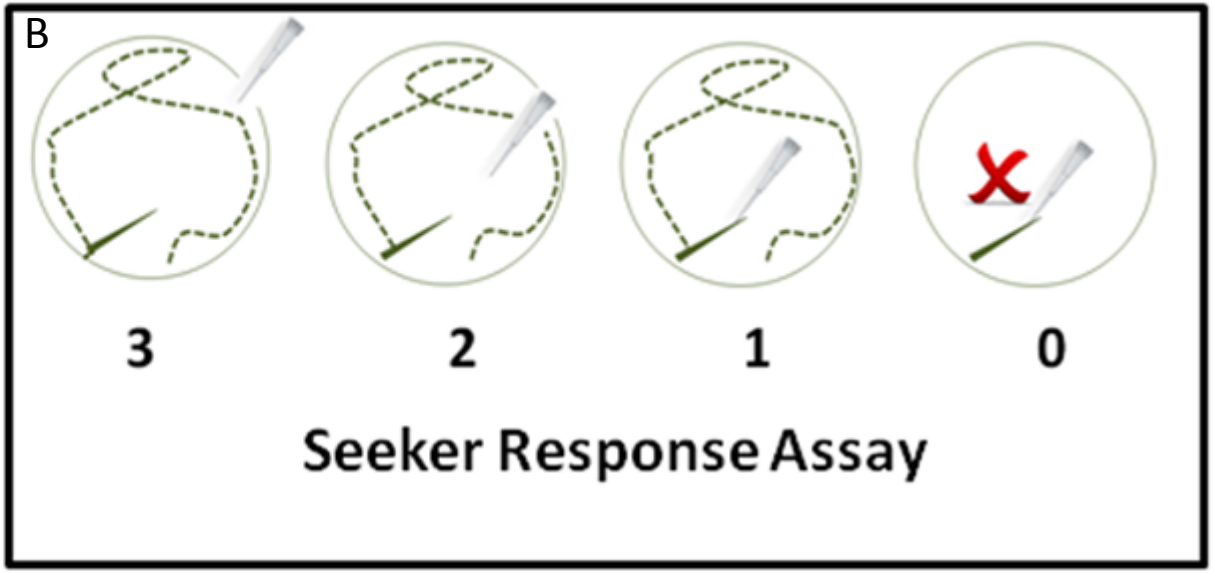




**Figure 2.7: Rheotaxis and seeker response testing.** Zebrafish larvae (5 dpf) were tested for rheotaxis ability and evasion responses before and after compound exposure. (A) Rheotaxis assay performed as described in Section 2.10.1. (B) Seeker response assay performed as described in Section 2.10.2.

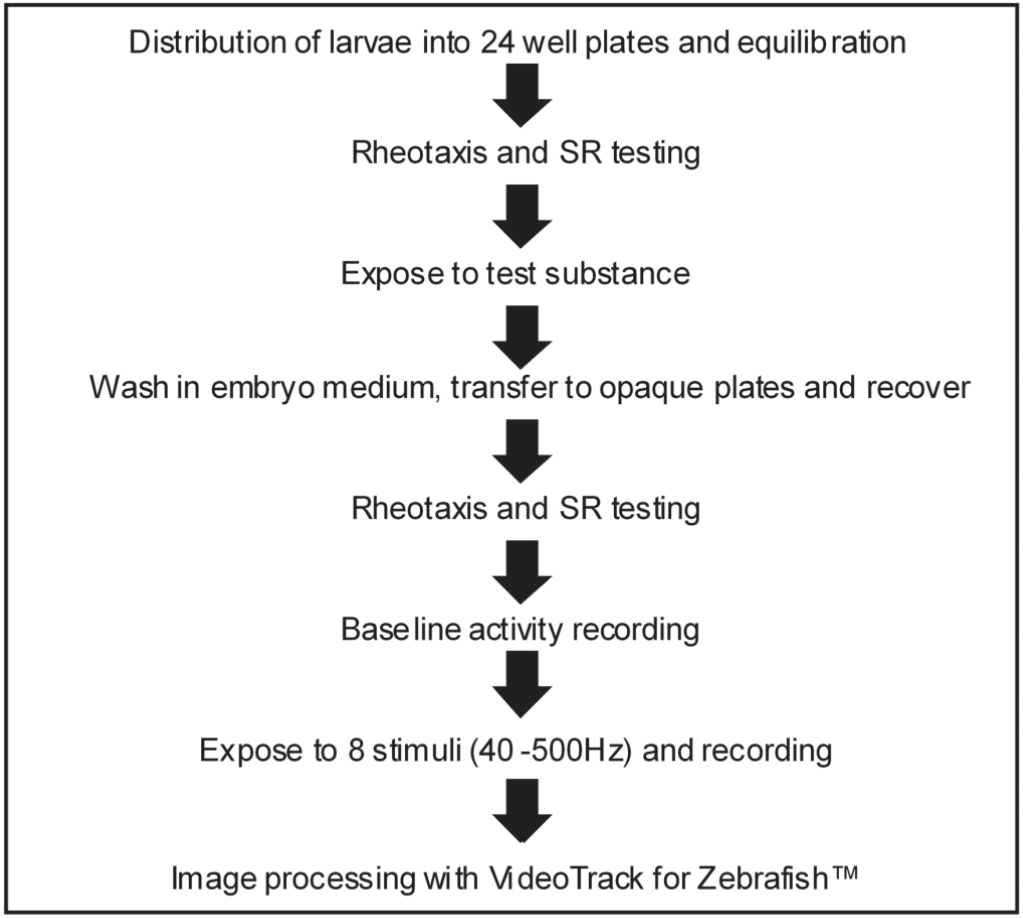



**Figure 2.8: Treatment and testing paradigm used in the startle, SR and rheotaxis assays.**

dechorionated using forceps prior to fixation. Following fixation, larvae were transferred by a PBS: methanol dilution series into 100% methanol and stored at -20ºC. For staining, larvae were rehydrated by rinsing in a methanol: 1X PTW dilution series (PTW: PBS plus 0.1% TWEEN® 20 (Sigma; CAS No. 9005-64-5)).

## 2.15 Detection of apoptosis

Apoptosis was detected by the Terminal Deoxynucleotide Transferase dUTP Nick End Label (TUNEL) method, using the ApopTag® Red *in Situ* Apoptosis Detection Kit (Millipore). Rehydrated samples (Section 2.14) were permeabilised by incubation in a 1:1000 dilution of Proteinase K solution (10mg/mL) for 2 hours. Digestion was stopped by rinsing the samples twice for 5 minutes in PTW. Samples were re-fixed in 4% PFA for 20 minutes at room temperature and then rinsed three times for 5 minutes with PTW. An 'acetone crack' step was performed by incubation of samples in 1 mL of cold 1:2 (acetone: ethanol) for 7 minutes at -20ºC. Following cracking, tubes were rinsed three times in PTW. Larvae were then incubated in 50 µL equilibration buffer for 1 hour at room temperature. The equilibration solution was removed and 16 µL terminal deoxynucleotidyl transferase (TdT) enzyme and 30 µL of Reaction Buffer was added to the tubes for 90 minutes at 37ºC. Following incubation, all the liquid was removed from the tubes and the enzymatic reaction was terminated by adding 200 µL STOP buffer for 3 hours at 37ºC. Larvae were then rinsed three times for a total of 15 minutes in PTW. To fluorescently label the embryos, 62 µL of Anti-Digoxigenin Rhodamine and 68 µL of blocking solution were added to the tubes overnight at 4ºC. On the second day of the protocol, the stain was stopped by washing four times for 30 minutes in PTW and the larvae were post-fixed at room temperature for 30 minutes in 4% PFA. Four final washes in PTW were performed to remove the fixative solution. TUNEL stained larvae were mounted in 80% glycerol: PTW solution and stored at -20ºC, ready for mounting and imaging at a later date (Section 2.2).

## 2.16 Data collection and statistics

For the DASPEI assay and mechanistic studies, data were collected manually, processed in Microsoft Excel and all raw data analysed using Prism 5.0 (GraphPad Software Inc., USA). For the startle assay and optimisation, data

were collected by camera recording and Viewpoint tracking, as previously described. Raw data from the Viewpoint software were then processed using macros written in Microsoft Excel (Appendix 1), and analysed using Prism 5.0.

Datasets were assessed for deviations from a normal distribution using the D'Agostino-Pearson normality test, and then for equality of variances using Bartlett's statistic. If tests indicated that a dataset deviated from a Gaussian distribution or had unequal variances, non-parametric methods were applied (e.g. Kruskal-Wallis and Dunn's tests). For all other datasets, parametric methods were appropriate (e.g. ANOVA).

In all analyses, I adopted a threshold value of 0.05α as statistically significant. In all cases, data are presented as the mean, with error bars plotted as the standard error of the mean (SEM).

# Chapter 3 Histological analysis of known human ototoxins in the zebrafish pLL

## Introduction

## 3.1 Aim

The aim of this study was to investigate the pathological consequences of in vivo exposure of larval zebrafish lateral line hair cells to a range of human ototoxins. The compound set tested comprised several known ototoxins, one lateral line (hair and support cell) toxin and several additional compounds that were not known to cause hair cell damage in mammals or fish.

## 3.2 Compounds: hair cell toxins

### 3.2.1 Aminoglycosides

#### 3.2.1.1 Streptomycin

The first of the aminoglycosides to be discovered was streptomycin in 1943 (Schatz et al., 2005). It is produced by the actinomycete *Streptomyces griseus* and has strong bactericidal action against both gram negative and positive infections. Streptomycin is primarily vestibulotoxic, with some auditory toxicity.

#### 3.2.1.2 Neomycin sulphate

Neomycin was first discovered in 1949 (Waksman and Lechevalier, 1949). It is produced naturally by the actinomycete *Streptomyces fradiae* and has strong bactericidal action against gram negative and some gram positive infections. Neomycin is administered topically and orally and is commonly used to reduce the bacterial population of the large intestine prior to bowel surgery, in skin graft operations and also in hepatic failure (Joint Formulary Committee, 2011). Neomycin in considered one of the most ototoxic compounds, and preferentially damages auditory hair cells (Kotecha and Richardson, 1994; Owens et al., 2009; Rotstein and Mandell, 2004; Wang et al., 1984).

#### 3.2.1.3 Gentamicin

Gentamicin was discovered in 1963 and is produced from a mixture of two species of *Micromonospora, M. pupurea* and *M. echinospora* (Weinstein et al., 1963). It is the most commonly employed aminoglycoside in the UK and is used

for numerous applications including the treatment of: meningitis and pneumonia, *E. coli*, *Staphylococcus*, urinary tract and bone infections. More recently, it has been used in the treatment of cystic fibrosis. Due to the vestibulotoxic effects of gentamicin, it can also be delivered by intratympanic injection to treat Ménière's disease (Joint Formulary Committee, 2011).

### 3.2.2 Cisplatin

Cisplatin was first approved for use by the FDA in 1978 and is the most ototoxic of all the platinum chemotherapeutics. It is administered intravenously to treat testicular, lung, cervical, bladder and head and neck cancer (Joint Formulary Committee, 2011; Rosenberg, 1985). Cisplatin causes permanent high frequency hearing loss over time, with preferential loss of the outer hair cells of the basal turn of the cochlea (Sturgeon, 2004).

### 3.2.3 Aspirin

Aspirin, an acetyl derivative of salicylic acid, is one of the most commonly used medicines worldwide. It was synthesized at the company Bayer in 1897 and was first marketed in 1899 (Sneader, 2000).

Aspirin is administered orally, rectally and topically to treat mild to moderate pain, fever and inflammation. It also has anti-platelet aggregating activity and is thus used in the treatment of transient ischemic attack, stroke and myocardial infarction (Joint Formulary Committee, 2011). Aspirin functions by irreversibly inhibiting cyclooxygenase enzymes COX-1 and COX-2 (Vane, 1971; Vane and Botting, 2003).

Aspirin and other salicylate derivatives have long been reported to have ototoxic effects. Most commonly, aspirin induces bilateral (flat or high frequency) hearing loss which is reversible and often preceded by tinnitus. Aspirin-induced tinnitus tends to be tonal and at frequencies of around 7-9 Hz, with a loudness of between 20 and 60 dB SPL. Recovery from the ototoxic effects of aspirin can take anywhere between one day and several weeks (Cazals, 2000; Prepageran and Rutka, 2004).

### 3.2.4 Copper sulphate

The death of hair cells is known to be induced by exposure to a variety of environmental factors, including heavy metals; methyl mercury and lead are

both documented to have ototoxic effects in humans (Prepageran and Rutka, 2004).

In recent years another heavy metal, copper, has been shown to cause hair cell toxicity in zebrafish (Hernandez et al., 2006; Linbo et al., 2006; Olivari et al., 2008). Copper is an important nutrient but it can be toxic to most cells at high exposure levels. High levels of dissolved copper (copper sulphate) from water can accumulate in aquatic organisms such as fish, leading to systemic toxicity (Clearwater et al., 2002; Grosell et al., 2003). Although copper ototoxicity has not been reported in humans, the mechanisms of ototoxin-induced hair cell death seen with copper partly mimic those caused by the aminoglycosides and cisplatin. Copper sulphate exposure elicits increased reactive oxygen species, neutrophil recruitment and apoptotic cell death (d'Alençon et al., 2010; Hernandez et al., 2006; Olivari et al., 2008).

### 3.2.5 Furosemide

Furosemide is a commonly used loop diuretic that is administered orally or intravenously to treat congestive cardiac failure, renal failure, cirrhosis and hypertension (Joint Formulary Committee, 2011). The hearing loss caused as an adverse effect of furosemide treatment is commonly transient, although some permanent cases of hearing loss have been recorded (Prepageran et al., 2004).

## 3.3 Compounds: Negative control compounds

A series of negative control compounds were selected based on similarities of therapeutic action with some of the ototoxic compounds listed above. Importantly, these negative control compounds had no published reports of impaired auditory or vestibular function associated with their use. These compounds included two antibiotics and two cytotoxic chemotherapeutic agents.

### 3.3.1 Amoxicillin

Amoxicillin is an orally administered semi-synthetic antibiotic of the aminopenicillin class.

The bactericidal activity of amoxicillin is explained by its ability to interfere with interpeptide linking of peptidoglycan, a structural molecule present in the cell

wall of bacteria. Without functional peptidoglycan, the cell wall is weakened and the bacteria undergo lysis upon division. Non-lytic bacterial death can also occur via changes in the bacterial membrane that collapse the membrane potential (Brunton et al., 2006).

Amoxicillin is primarily used to treat bacterial meningitis and upper respiratory tract, urinary tract, and salmonella infections. It is additionally employed to treat infection of the inner ear (Joint Formulary Committee, 2011).

Commonly reported side-effects of this compound are nausea, vomiting, diarrhoea and rashes. Rarely, antibiotic-associated colitis is encountered (Joint Formulary Committee, 2011).

### 3.3.2 Cefazolin

Cefazolin is a semi-synthetic first-generation antibiotic of the cephalosporin class. It has a similar bactericidal activity to that of amoxicillin (Brunton et al., 2006).

Cefazolin is usually administered by intravenous injection and is used to treat skin and soft tissue infections, and as prophylaxis for surgery where skin infection is likely. It is particularly active against *Staphylococcus aureus* and *Streptococcus pyogenes.*

The most common side-effect of cefazolin is hypersensitivity. Other side-effects include loss of appetite, diarrhoea, nausea and vomiting. Rarely, colitis and nephrotoxicity occur (Joint Formulary Committee, 2011).

### 3.3.3 Melphalan

The chemotherapeutic melphalan is the phenylalanine derivative of nitrogen mustard. Similar to cisplatin, melphalan is an alkylating agent. It acts in a comparable manner, binding DNA covalently by nucleophilic attack (Brox et al., 1980; Ross et al., 1978).

Melphalan is administered both orally and intravenously and is used in the treatment of malignant melanoma of the extremities, localised soft-tissue sarcoma of the extremities, multiple myeloma, *polycythaemia vera* and childhood neuroblastoma.

The main toxicity of melphalan is haematological, causing a decrease in red and white blood cells and platelets. These effects lead to anaemia, infections and bruising. Nausea, diarrhoea and infertility are also commonly observed (Joint Formulary Committee, 2011).

### 3.3.4 Gemcitabine

Gemcitabine is a member of the antimetabolite class of chemotherapeutics acting to inhibit DNA function. Specifically, gemcitabine is transported into dividing cells and converted into the active cytidine analogues difluorodeoxycytidine diphosphate (dFdCDP) and difluorodeoxycytidine triphosphate (dFdCTP). Once converted, dFdCTP can incorporate into the DNA, inhibiting the elongation of growing DNA strands. Additionally, dFdCTP inhibits DNA polymerase activity, reportedly poisons topoisomerase I and may bind to RNA (Mini et al., 2006).

Gemcitabine hydrochloride is administered intravenously to treat pancreatic cancer. It is also used in adjuvant therapy with cisplatin to treat locally advanced or metastatic non-small cell lung cancer and advanced bladder cancer.

The most common off-target effects of gemcitabine are: musculoskeletal pain, mild gastro-intestinal effects, rashes and flu-like symptoms. Renal impairment and pulmonary toxicity are rarely encountered (Brunton et al., 2006).

## 3.4 Hair Cell visualisation

### 3.4.1 DASPEI

DASPEI (2-(4-(dimethylamino)styryl)-N-Ethylpyridinium Iodide) is a vital dye used as a mitochondrial label in situ (Bereiter-Hahn, 1976). In teleosts, it was initially used to study active chloride transport in the skin (Marshall and Nishioka, 1980). Since then, DASPEI has been harnessed extensively for labelling of hair cells in the zebrafish pLL to study lateral line mutations, hair cell regeneration and hair cell damage induced by ototoxins (Balak et al., 1990; Harris et al., 2003; Ton and Parng, 2005; Van Trump et al., 2010; Whitfield et al., 1996)

### 3.4.2 FM1-43FX

FM1-43FX is a fixable form of FM1-43 (*N*-(3-Triethylammoniumpropyl)-4-(4-(Dibutylamino)styryl)Pyridinium Dibromide). It is a lipophilic styryl vital dye with a four carbon tail that provides bright signal when dissolved in a membrane (Betz et al., 1996). The dye is taken up by two mechanisms: rapidly via mechanotransduction and ion channels and more slowly by endocytosis (Gale et al., 2001; Meyers et al., 2003; Nishikawa and Sasaki, 1996). In hair cells, FM1-43 labels the cytoplasm including the endoplasmic reticulum and mitochondria. In support cells, the dye only labels the plasma membrane. The rapid immersion time in this study means that uptake is most likely via mechanotransduction channels. Due to the rapid uptake of FM1-43 and the fixable nature of this dye, it can be used as an alternative to DASPEI to measure the health of hair cells.

### 3.4.3 DAPI

DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) is a trypanocide which fluoresces blue upon binding to A-T rich regions of dsDNA (Dann et al., 1971; Kubista et al., 1987). In zebrafish, DAPI has been used to stain nuclei of fixed and live embryos, including live hair cell nuclei (Kane et al., 1996; Olivari et al., 2008).

### 3.4.4 TUNEL

The terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay is a method of labelling DNA breaks in nuclei (Gavrieli et al., 1992). Specifically, it has been used as a marker of programmed cell death and late stage apoptotic cells. Fragmentation of DNA is associated with changes in cellular morphology during apoptosis and is a consequence of the action of endonucleases. The TUNEL method used in this chapter is based on the specific binding of the terminal deoxynucleotidyltransferase (TdT) enzyme to 3-OH ends of the fragmented DNA. The assay hijacks this by using TdT to incorporate digoxigenin-labelled dUTP (and non-labelled) nucleotides. Labelled nucleotides can be detected by immunofluorescence by an anti-DIG antibody with a rhodamine fluorochrome. The TUNEL assay has been used successfully for a number of applications in zebrafish (e.g. Elks et al., 2011; Lu et al., 2011).

## 3.5 *Tg(pou4f3::mGFP)s356t* transgenic larvae

The *(pou4f3::mGFP)s356t* transgenic line was generated in 2005 in order to study axon guidance in a distinct subset of zebrafish retinal ganglion cells (RGCs). This GFP reporter line additionally labels mechanosensory hair cells and was therefore useful in studying the effects of ototoxins on hair cells of the ear and lateral line (Xiao et al., 2005).

## 3.6 *i193* transgenic larvae

A BAC transgenic line was made (Stone Elworthy, Fredericus van Eeden and Philip Ingham; unpublished line) to rescue the smoothened genotype in larval zebrafish. In this line (*i193*), the construct is a BAC with the genomic *smo* gene that has an epitope tag on the C-terminus of the smo coding sequence. A heatshock:GFP that is unrelated to the *smo* gene is present in the BAC vector to provide a visible marker so as to facilitate generation and maintenance of the transgenic line. Coincidentally, GFP is also expressed in the hair cells of the ear and lateral line. This expression is likely to be due to random insertion of the transgene into the genome, which happened by chance, directing GFP expression to the hair cells.

### Methods

The techniques described in Chapter 2 were used to build simple profiles of hair cell toxicity for each individual compound. For full methods, see Sections 2.1-2.8 and Sections 2.14-2.16.

### Results

## 3.7 Determination of MTC levels for the acute exposure of larvae to ototoxic compounds

For the purposes of subsequent experiments, it was necessary to determine the maximum tolerated concentration (MTC) before larval mortality and to build a concentration-response profile for each compound tested. This required the optimisation of a simple, fast and reliable technique to determine mortality. Thus, the maximum tolerated concentration level was approximated. This was the concentration to elicit more than twenty percent death following compound exposure. MTC data obtained provided a basis for subsequent compound exposure ranges for the DASPEI fluorescence assay (see Table 2.1). In the

majority of cases, systemic toxicity requires good uptake of the exposure compound. For hair cell damage such compound uptake is not required, as the hair cells of the lateral line are in almost direct contact with the compound in solution.

### 3.7.1 Neomycin

The MTC of neomycin was approximated to be 300 µM after an initial exposure (minimum of 9 fish per concentration). The treatment point that resulted in more than 20% mortality was 500 µM (Figure 3.1).

### 3.7.2 Streptomycin

The MTC of streptomycin was approximated to be 3.5 mM after the first exposure (minimum of 8 fish per concentration). Twenty percent mortality was exceeded at 4 mM (Figure 3.1).

### 3.7.3 Gentamicin

A mortality rate of above 20% was not reached after an hour of gentamicin treatment, even at a concentration of 10 mM (minimum of 6 fish per group; Figure 3.1).

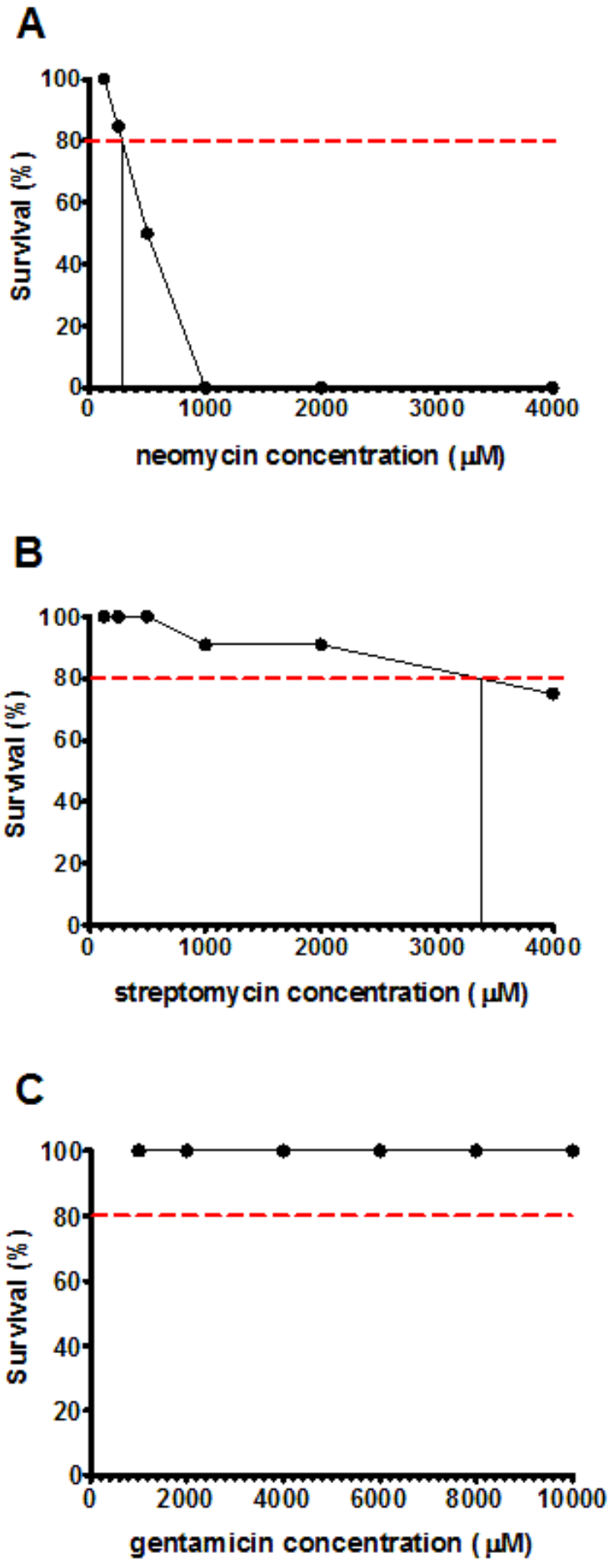



**Figure 3.1: Maximum Tolerated Concentration data for the aminoglycoside antibiotics following acute exposure.**

### 3.7.4 Cisplatin

An initial exposure to cisplatin for two hours failed to elicit more than 20% mortality at concentrations up to 1 mM (minimum of 9 fish per group). Interestingly, a second exposure from 1 mM to 10 mM showed that the concentration of 1 mM was highly toxic to animals (71% death; *n* = 6 per group). Overall, the MTC was taken as 1 mM (Figure 3.2).

### 3.7.5 Aspirin

The MTC of aspirin was approximated to be 1.1 mM after initial exposure (minimum of 7 fish per group). Twenty percent mortality was exceeded at 2 mM in the initial test (Figure 3.3). A second test at 1.5 mM showed that 100% mortality could also be reached at this level (data not shown).

### 3.7.6 Copper sulphate

The MTC of copper sulphate was approximated to above 140 µM after initial exposure (minimum of 7 fish per group). The treatment point that resulted in more than 20% mortality was 250 µM in the initial test (Figure 3.3). A second test at concentrations of 150 and 200 µM showed that > 20% mortality could also be reached at these levels (data not shown).

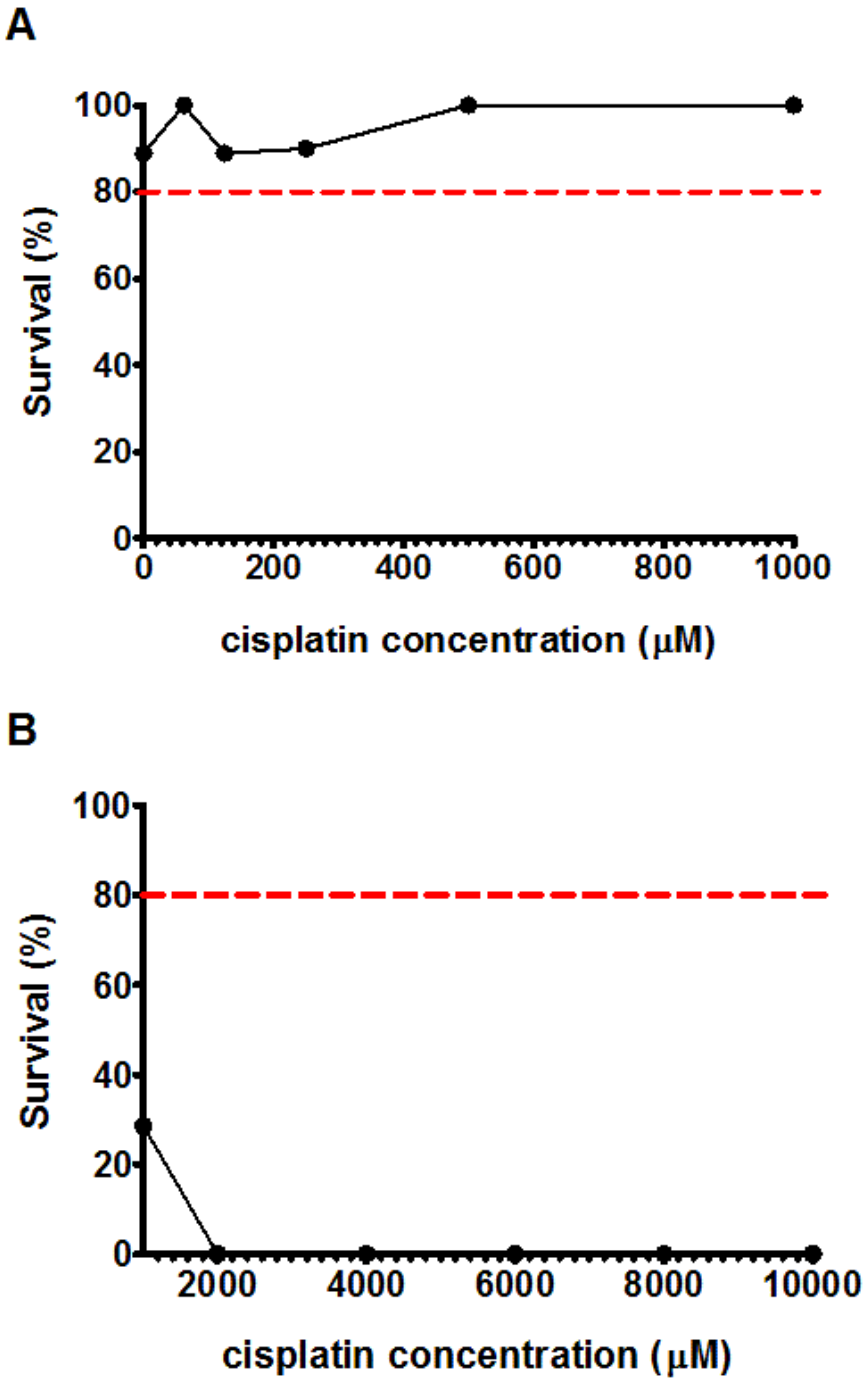



**Figure 3.2: MTC data obtained for acute cisplatin exposure over two trials shows variable mortality thresholds.**

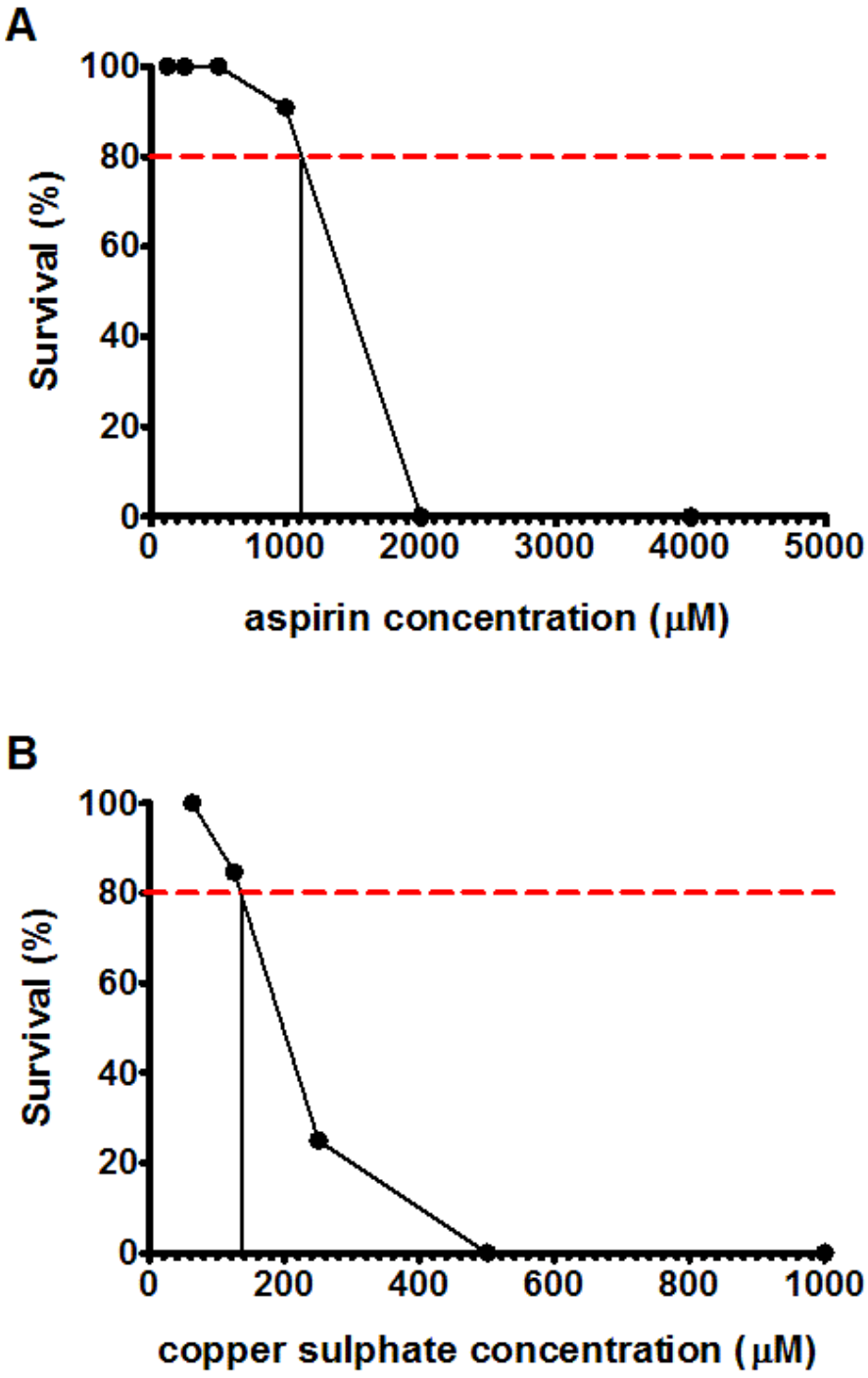



**Figure 3.3: Maximum Tolerated Concentration data for acute exposure to aspirin and copper sulphate.**

### 3.7.7 Furosemide

The MTC of furosemide was highly variable following a two hour exposure. MTC ranged from 1mM to 5 mM and was probably linked to the variable solubility of the compound in solution (minimum of 8 larvae per treatment group, data not shown). The maximum exposure concentration ever used following MTC assessment was 1 mM.

## 3.8 Determination of MTC levels for the acute exposure of larvae to negative control compounds

### 3.8.1 Antibiotics

#### 3.8.1.1 Amoxicillin

Initial exposures suggested that the MTC was above 5 mM, re-testing was carried out at a higher concentration range. In the second test, MTC was estimated at 11.2 mM (minimum of 6 fish per concentration). The treatment point that resulted in more than 20% mortality was 12 mM (Figure 3.4).

#### 3.8.1.2 Cefazolin

A mortality rate of above 20% was not reached after an hour of cefazolin treatment, even at a concentration of 20 mM (8 fish per treatment group; Figure 3.4).

### 3.8.2 Chemotherapeutics

#### 3.8.2.1 Melphalan

The MTC of melphalan was approximated to 820 µM (6 fish per concentration). The treatment point that resulted in more than 20% mortality was 1 mM (Figure 3.4).

#### 3.8.2.2 Gemcitabine

The MTC of gemcitabine was approximated to be 1.4 mM after an exposure of 30 minutes (6 fish per concentration). The treatment point that resulted in over 20% mortality was 1.5 mM (Figure 3.4).

## 3.9 Vital dye staining of the mitochondria of live hair cells following acute compound exposure

### 3.9.1 Ototoxins affect mitochondrial DASPEI staining in the pLL

The mitochondrial vital dye DASPEI was used to investigate the effects of ototoxins on the posterior lateral line. DASPEI staining was reduced by treatment with all except one of the human ototoxins tested, suggestive of damage to the hair cells of the pLL. An almost complete reduction in DASPEI staining was observed for all three aminoglycosides, cisplatin and copper sulphate at higher concentrations. Aspirin also strongly reduced the DASPEI stain in association with increasing treatment concentration. Furosemide exposure did not reliably alter the DASPEI staining. The nasal epithelium staining seen with DASPEI immersion was not affected by exposure to any of the human ototoxins. Labelling of the nasal epithelium therefore acted as an internal control for the reliability of the DASPEI staining method. Additionally, it was observed that the posterior lateral line seemed to be more susceptible to hair cell damage than the anterior lateral line at mid-concentration ranges. This observation did not warrant further investigation, as my primary interest was in the pLL. Representative images of DASPEI staining in control versus treated animals are shown in Figure 3.5 (a minimum of 12 larvae were stained for each treatment condition).

The negative control compounds amoxicillin, cefazolin and melphalan did not cause a visible reduction in DASPEI staining (Figure 3.5). This result indicated that the compounds were not toxic to the hair cells of the pLL. Surprisingly, gemcitabine caused a decrease in DASPEI staining above 50 µM (Figure 3.16). This result meant that gemcitabine was not used in further testing.

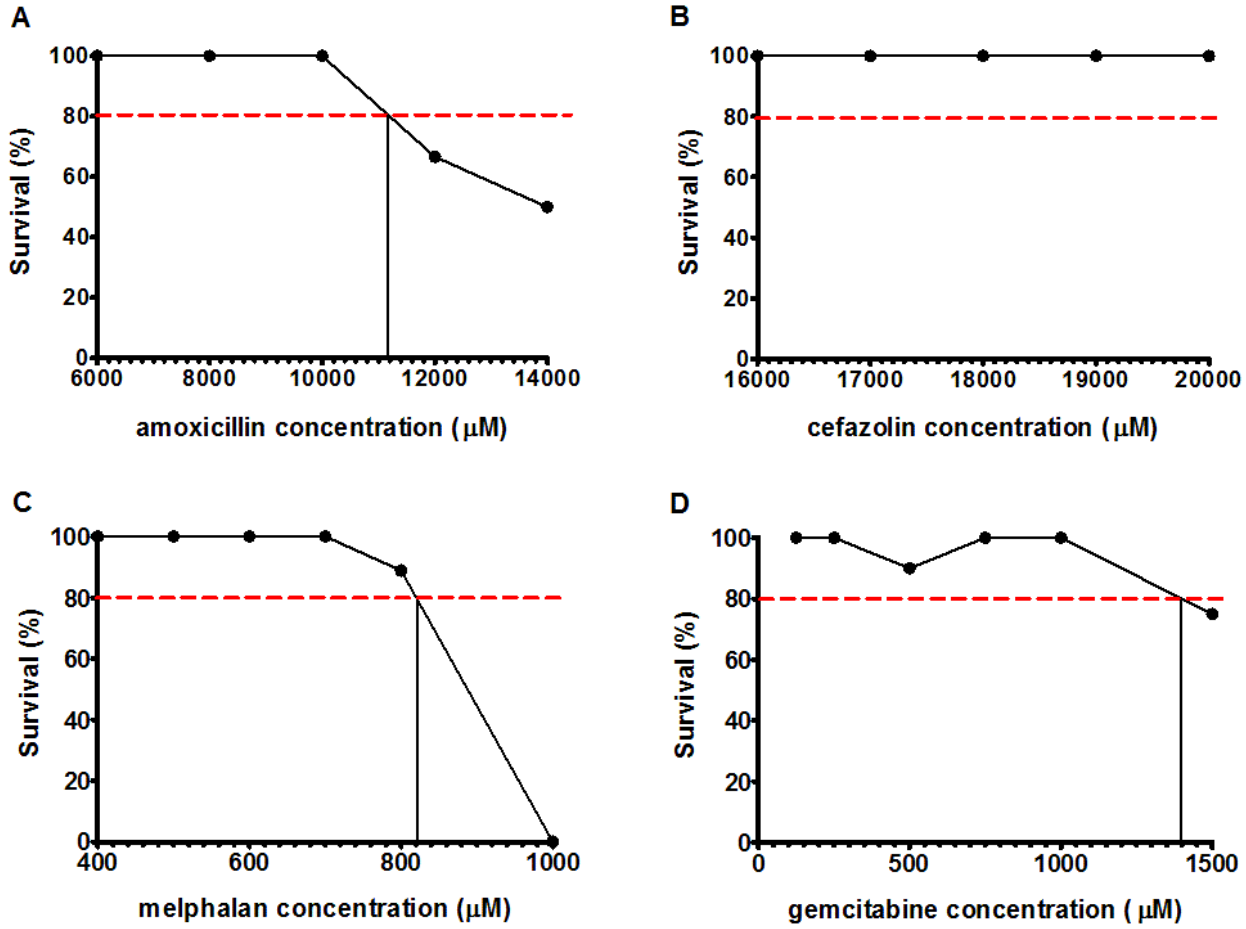



**Figure 3.4: Maximum Tolerated Concentration data for acute exposure to the negative control compounds.**

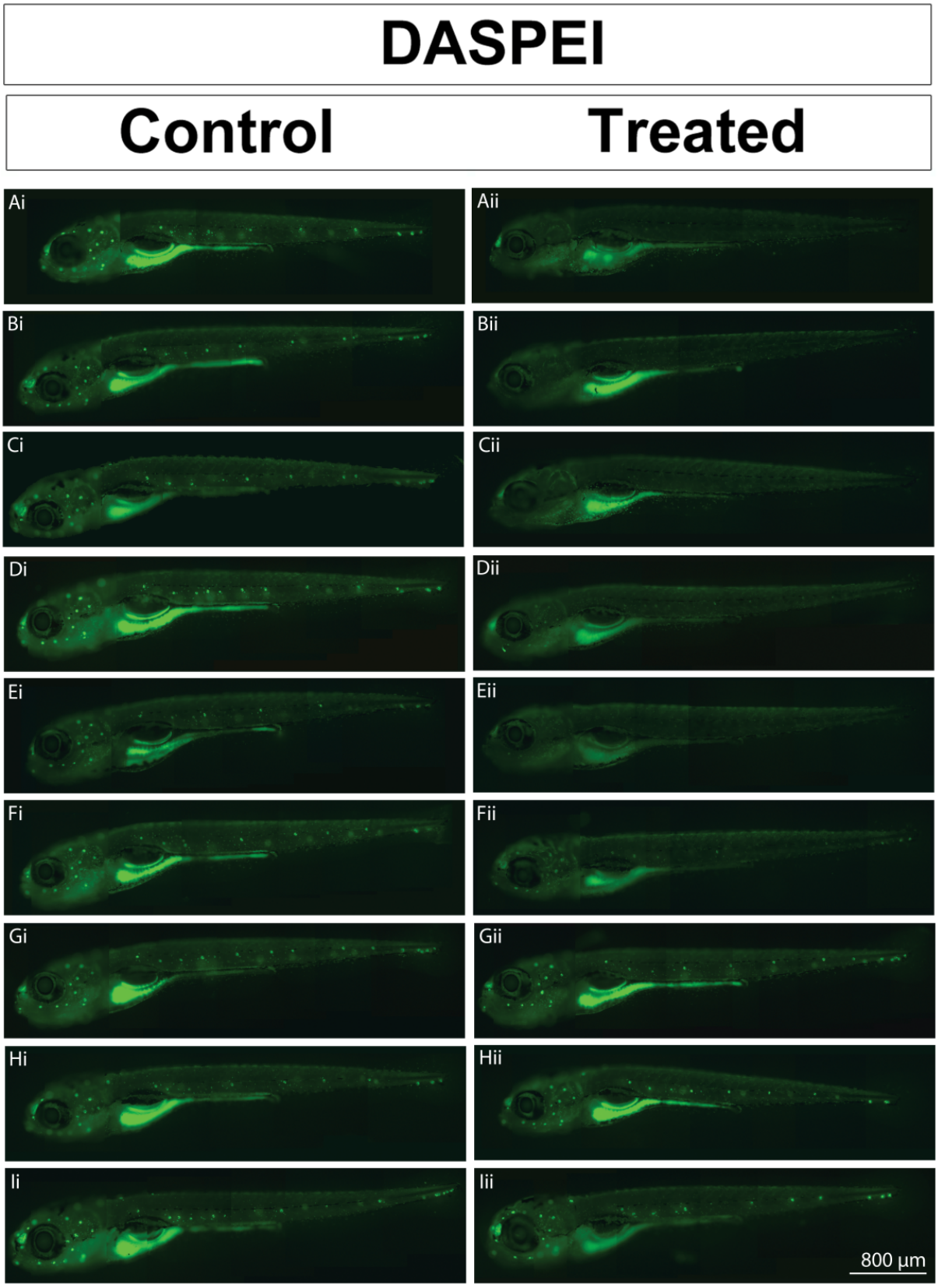



**Figure 3.5: The effects of compounds on DASPEI staining in the pLL.** Ototoxin treatment reduced DASPEI staining in the hair cells of the pLL compared to control larvae. Row A = neomycin; Row B = streptomycin; Row C = gentamicin; Row D = cisplatin; Row E = copper sulphate; Row F = aspirin; Row G = amoxicillin; Row H = cefazolin; Row I = melphalan. Staining in the nasal epithelium was not affected in treated larvae (rows A-F). Treatment with negative control compounds did not alter the brightness of DASPEI labelling in the pLL compared to controls (rows G-I). Images are representative of 12 larvae per treatment group over a minimum of 3 trials.

## 3.10 Vital dye staining for the mechanotransductive activity of live hair cells following acute compound exposure

### 3.10.1 Ototoxins affect FM1-43FX staining in the pLL

The vital dye FM1-43FX was used to further investigate the effects of ototoxins on the posterior lateral line and to confirm what was observed using DASPEI staining. A reduction in labelling was taken to be indicative of reduced mechanotransduction in the cells, and therefore cell damage. This stain was indicative of the uptake activity of the healthy hair cell. FM1-43FX staining was reduced by treatment with all of the human ototoxins tested except one, suggestive of damage to the hair cells of the pLL. A strong reduction in FM1-43FX stain was observed for all three aminoglycosides, cisplatin and copper sulphate at higher doses. Aspirin did not appear to reduce the FM1-43FX staining at the top concentration; this suggested some hair cell function was retained. Overall, it appeared that DASPEI labelling was a more sensitive indicator of ototoxic damage, as it could detect aspirin-induced hair cell damage, whereas FM1-43 staining could not. Representative images of FM1-43FX staining in control versus treated animals are shown in Figure 3.6.

The negative control compounds amoxicillin, cefazolin and melphalan did not cause a visible reduction in FM1-43FX staining. This indicated that the compounds were not toxic to hair cells of the pLL.

## 3.11 Hair cell damage to the pLL in *Tg(pou4f3::mGFP)s356t* larvae following acute compound treatments

### 3.11.1 Ototoxins affect GFP fluorescence in the pLL

Following testing using vital dyes, I next investigated the effects of the test compounds in transgenic larvae. This focussed on an assessment of GPF fluorescence in pLL hair cells in the *Tg(pou4f3::mGFP)s356t* line. A reduction in fluorescence was expected to be indicative of hair cell damage that would correlate with reduced vital dye staining. GFP fluorescence was visibly reduced by treatment with all of the human ototoxins tested except copper sulphate, where GFP fluorescence did not appear reduced (Figure 3.7). This indicated the hair cells were damaged but not destroyed by treatment with copper sulphate.

In all cases of acute ototoxic treatment there was no decrease in the GFP fluorescence within the ears of the larvae and no change in the morphology of the inner ear structures (maculae and cristae), suggesting that the compounds could not access the inner ear sufficiently to induce visible damage (see Figure 3.18). Long term exposure to compounds at lower doses for 24 hours also had no effect on inner ear GFP fluorescence.

In agreement with vital dye labelling assays, the negative control compounds amoxicillin, cefazolin and melphalan did not cause an observable reduction in GFP fluorescence. This suggested that the compounds were not toxic to hair cells of the pLL.

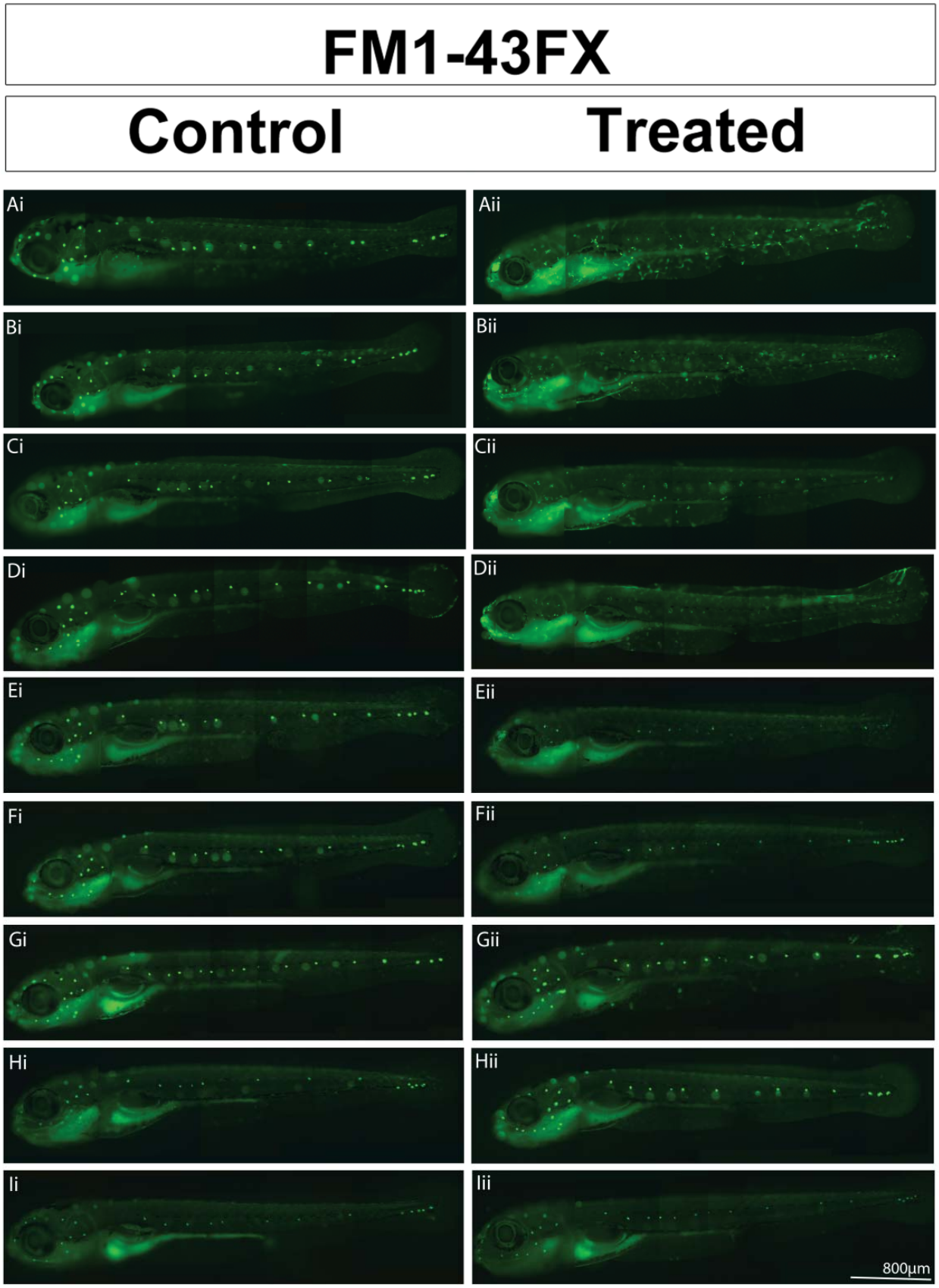



**Figure 3.6: The effects of compounds on FM1-43FX staining in the pLL.** Acute treatment with ototoxins (except aspirin) reduced FM1-43FX staining in the hair cells of the pLL compared to control animals. Row A = neomycin; Row B = streptomycin; Row C = gentamicin; Row D = cisplatin; Row E = copper sulphate; Row F = aspirin; Row G = amoxicillin; Row H = cefazolin; Row I = melphalan. Staining of the nasal epithelium acted as an internal control for treated animals (rows A-F). Treatment with negative control compounds did not visibly change FM1-43FX labelling in the pLL compared to control larvae (Rows G-I). The images shown are representative of all fish tested (12 larvae per treatment group).

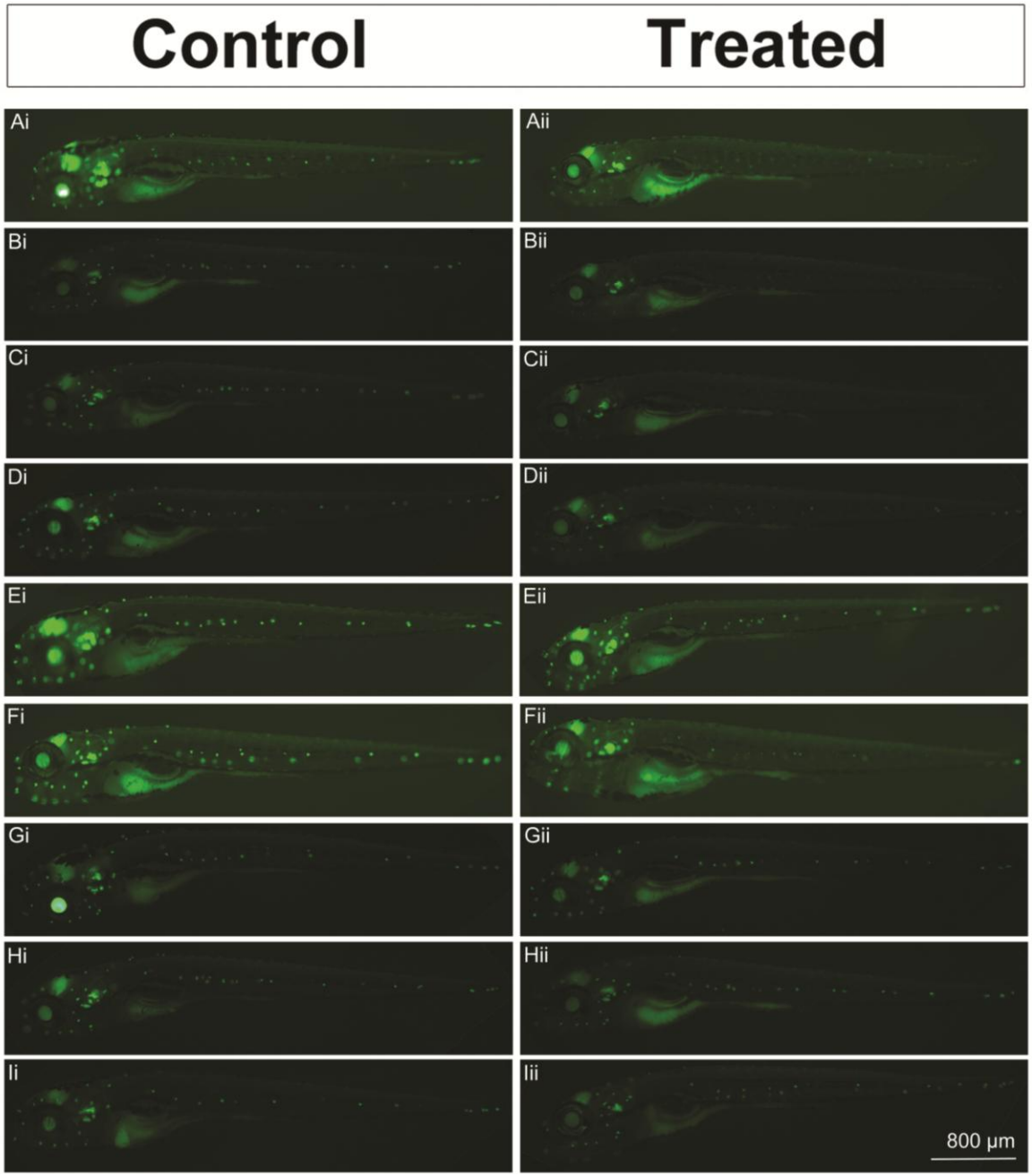



**Figure 3.7: The effects of compounds on GFP expression in the pLL of *Tg(pou4f3::mGFP)s356t* transgenic larvae.** Ototoxin treatment (except with copper sulphate) reduced GFP expression in the hair cells of the pLL compared to control larvae. Row A = neomycin; Row B = streptomycin; Row C = gentamicin; Row D = cisplatin; Row E = copper sulphate; Row F = aspirin; Row G = amoxicillin; Row H = cefazolin; Row I = melphalan. Staining in the ear was not affected in ototoxin treated larvae (rows A-F). Treatment with negative control compounds did not reduce the brightness of GFP in the pLL compared to control larvae (Rows G-I). Images displayed are representative of 12 larvae per treatment group.

## 3.12 Neomycin causes hair cell death: DAPI and TUNEL

### 3.12.1 DAPI

In order to show that the decrease in vital dye staining and GFP expression in live fish correlated with hair cell death in addition to hair cell damage, a quick method to assess alterations in cell morphology was established. Live cell labelling using the nuclear dye DAPI in control animals showed the individual nuclei of hair cells to have a large, rounded shape indicating a healthy cell. The DAPI stain also revealed the rosette arrangement of hair cells in the healthy neuromast. Very occasionally, nuclear condensation could be observed in a single cell within the cluster of hair cells (Figure 3.8 B arrowhead). In larvae treated with high concentration neomycin there were clear alterations in nuclear morphology (condensed and fragmented nuclei) and the rosette structure was lost (Figure 3.8 C, D). In agreement with previous studies in zebrafish, these changes appeared to be indicative of apoptosis and were observed not only for neomycin but also with other ototoxins, including cisplatin (data not shown).

### 3.12.2 TUNEL

The TUNEL assay was used to confirm that the changes in DAPI staining seen with neomycin treatment were a true indicator of apoptosis. As expected, TUNEL labelling was observed in areas of high cell turnover in both control and treated animals. In control larvae, there was no TUNEL labelling observed along the lateral line and the cells looked healthy under DIC microscopy. In larvae that had been treated with 100 µM neomycin, there was an increase in TUNEL staining in the neuromasts in all cases (Figure 3.9). This supported the view that changes in nuclear morphology observed using DAPI corresponded, at least in part, to apoptotic death. The possibility that some necrosis was also occurring following ototoxin treatment could not be excluded however.

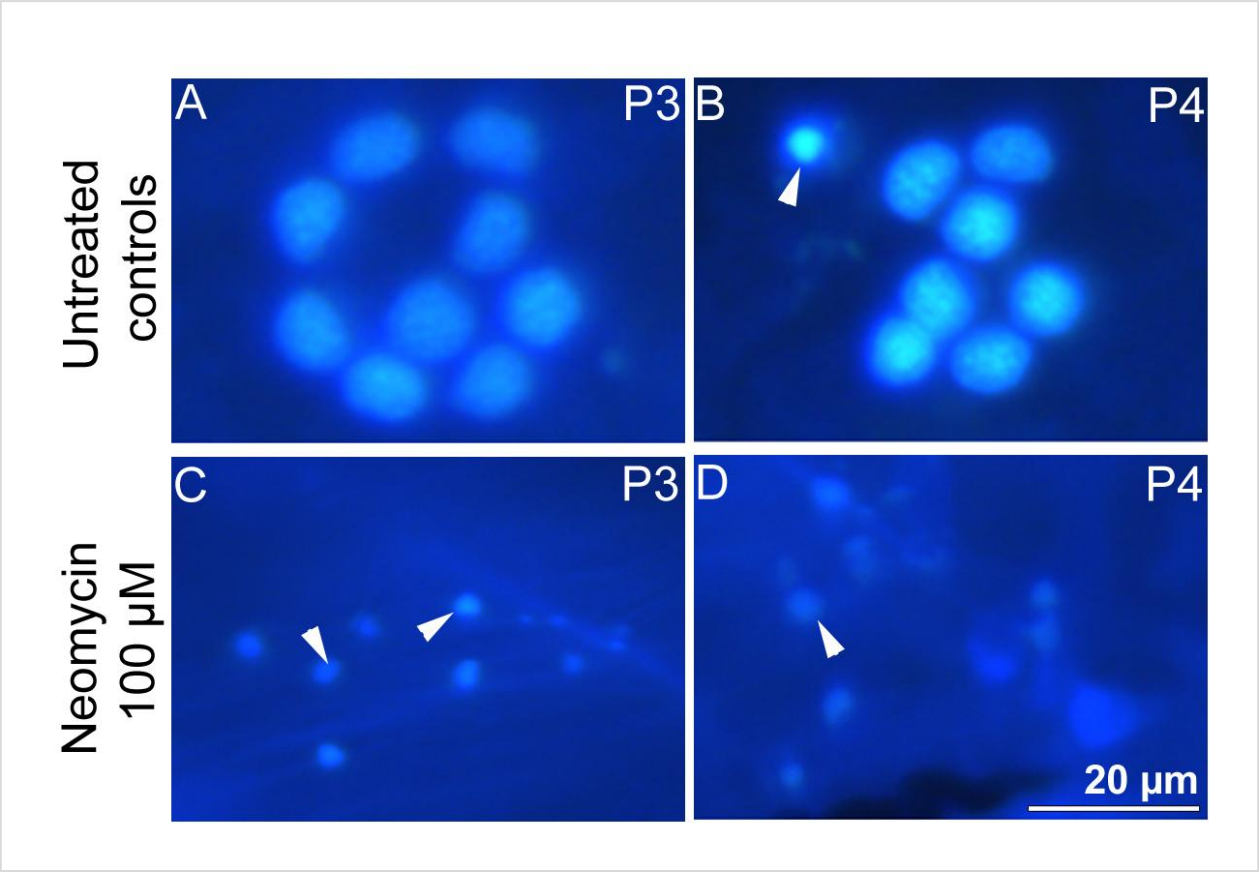



**Figure 3.8: Treatment of larvae with high-dose neomycin induces apoptotic-like changes in nuclear morphology that can be visualised by live DAPI staining.** (A, B) In neuromasts P3 and P4 of control (untreated) larvae, the majority of nuclei have a round morphology. A single condensed nucleus can be seen in panel (B) (arrowhead). In larvae treated with 100 µM neomycin for one hour (C, D), the majority of nuclei have condensed or fragmented into smaller bodies (some examples are indicated with arrowheads). The characteristic rosette-like morphology of the neuromast is no longer visible. These alterations are indicative of apoptosis. Images are representative of 12 larvae per treatment group.

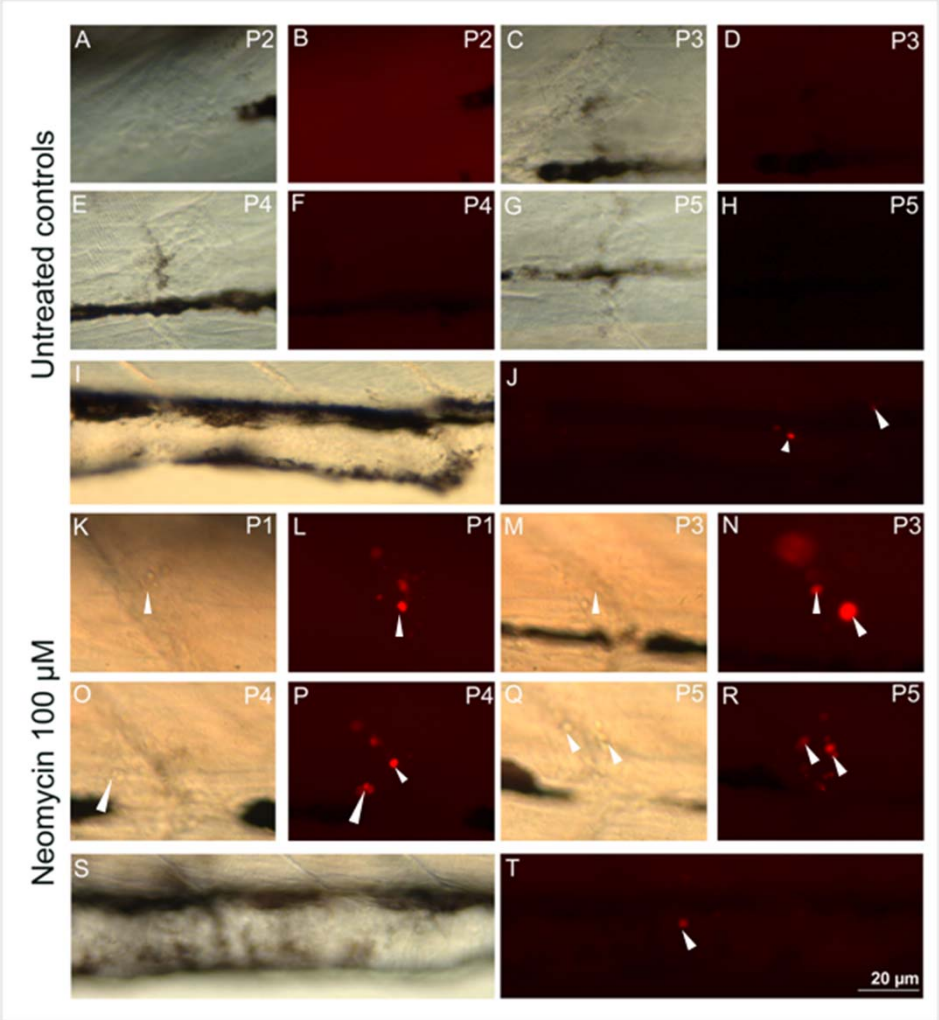



**Figure 3.9: Neomycin treatment increases TUNEL labelling in the neuromasts of the pLL.** In control larvae, the neuromasts look healthy under Differential Interference Contrast (DIC) microscopy and TUNEL staining is not visible (A-H). (K-R) Treatment with 100 µM neomycin for an hour with one hour recovery results in increased TUNEL staining in the neuromast, indicative of apoptosis. DIC microscopy also shows some altered cell morphology (arrowheads). As expected, TUNEL labelling was additionally observed in areas of high cell turnover in both control and treated larval samples (J, T) .

## 3.13 Optimisation of the DASPEI staining method to quantify hair cell damage

### 3.13.1 Duration of exposure to DASPEI affects the quality of staining

To optimise the DASPEI staining process, the duration of exposure of larvae to DASPEI was altered and the effects of staining time were observed (Figure 3.10). Exposure times varied from 0-30 minutes. Untreated larvae that were exposed to DASPEI for 5 minutes or less had significantly weaker staining than those exposed to the dye for 10 minutes or longer (Kruskal-Wallis test, $H$ = 110.7, 6 *d.f.*, $P < 0.0001$). There was no difference in staining score for any of the time points between 10 and 30 minutes (Dunn's multiple comparison test, $P > 0.05$). In all subsequent assays, larvae were immersed in DASPEI solution for 20 minutes to guarantee sufficient staining.

### 3.13.2 The use of anaesthesia has no effect on DASPEI staining

It was important to ensure that the use of anaesthesia to observe and score larvae did not affect the strength of DASPEI staining in control animals. Reduced temperature (by cooling the plate on ice) or reduced temperature plus MS222 were used to sedate the larvae and the strengths of staining scores were compared. The use of MS222 for 30 minutes or less had no significant effect on the average score of staining (Figure 3.10, Mann Whitney U test, MWU statistic = 348.5, 1 *d.f.*, $P = 0.4125$).

### 3.13.3 The effect of pH on DASPEI staining

#### 3.13.3.1 Untreated control larvae

Untreated larvae were immersed in embryo medium with different pH levels and the effects of pH on resultant DASPEI scores were observed (Figure 3.11). Results from two initial tests indicated that DASPEI scores could be affected by pH (test 1: $F$ (6, 28) = 8.951, $P$ <0.0001; test 2: $F$ (8, 33) = 26.28, $P$ < 0.0001). DASPEI scores were negatively affected at lower pH, but less affected at alkaline or neutral pH levels. A follow-up test on a range of more acidic solutions (3.83-6.03), suggested that DASPEI score could be affected by certain acidic conditions ($F$ (4, 44) = 6.118, $P$ = 0.0005). As a small subset of the compounds tested had a lower pH in water, it was necessary to carry out preliminary investigations to assess the effects of pH alteration in control versus treated larvae.

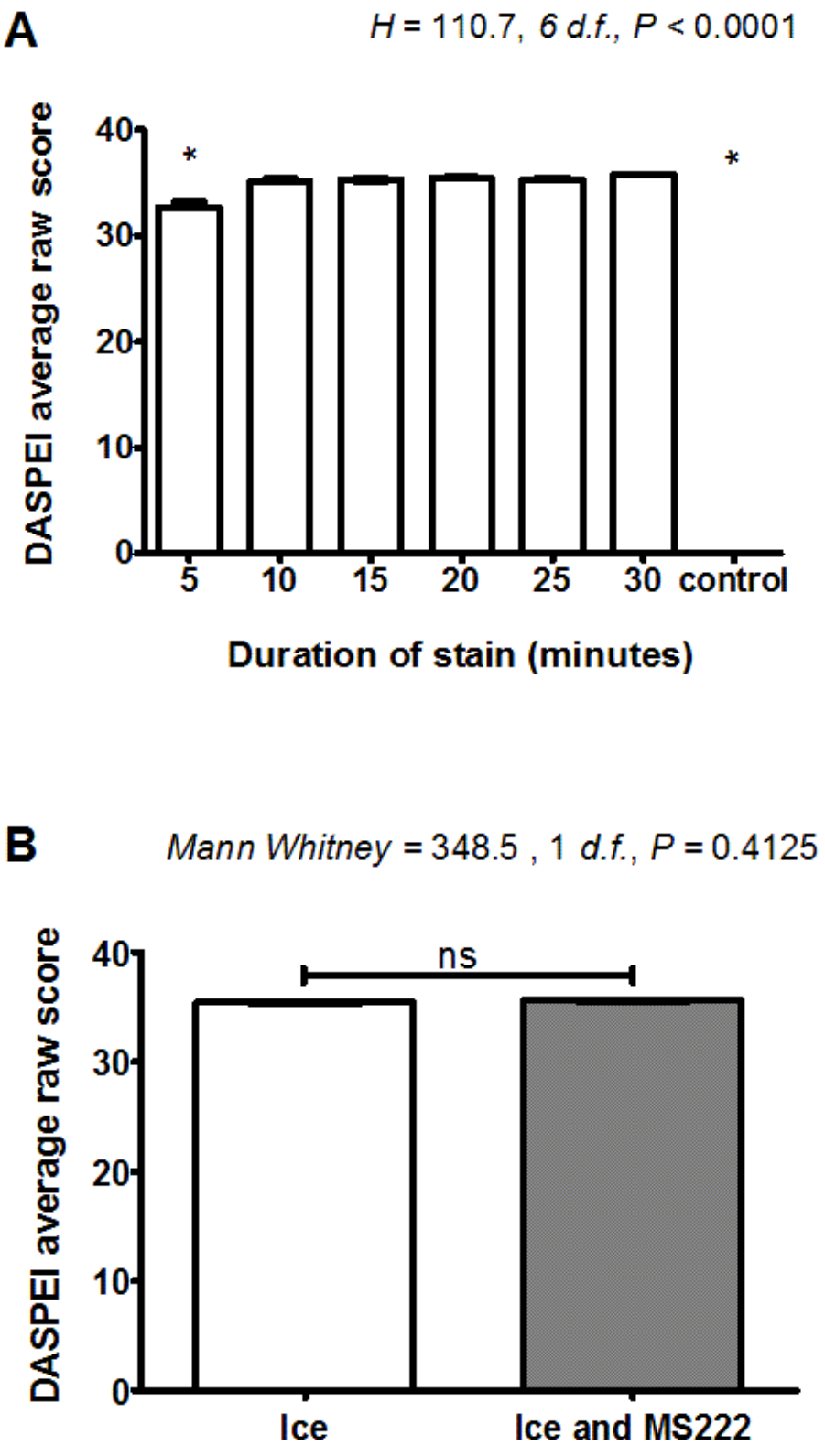



**Figure 3.10: The effects of duration of staining and anaesthetic treatment on DASPEI labelling.** (A) The duration of incubation with DASPEI significantly affects the DASPEI score (strength of fluorescence staining). Control larvae incubated for 10 minutes or longer have a significantly improved DASPEI score compared with larvae incubated for only 5 minutes ($n$ = 28 per group from 1 trial, Kruskal-Wallis test, $H$ = 110.7, 6 *d.f.*, $P$ < 0.0001; Dunn's multiple comparison post-test). (B) DASPEI score is unaffected by anaesthetic treatment ($n$ = 28 per group over 1 trial , Mann Whitney U test, MWU statistic = 348.5, 1 *d.f.*, $P$ = 0.4125).

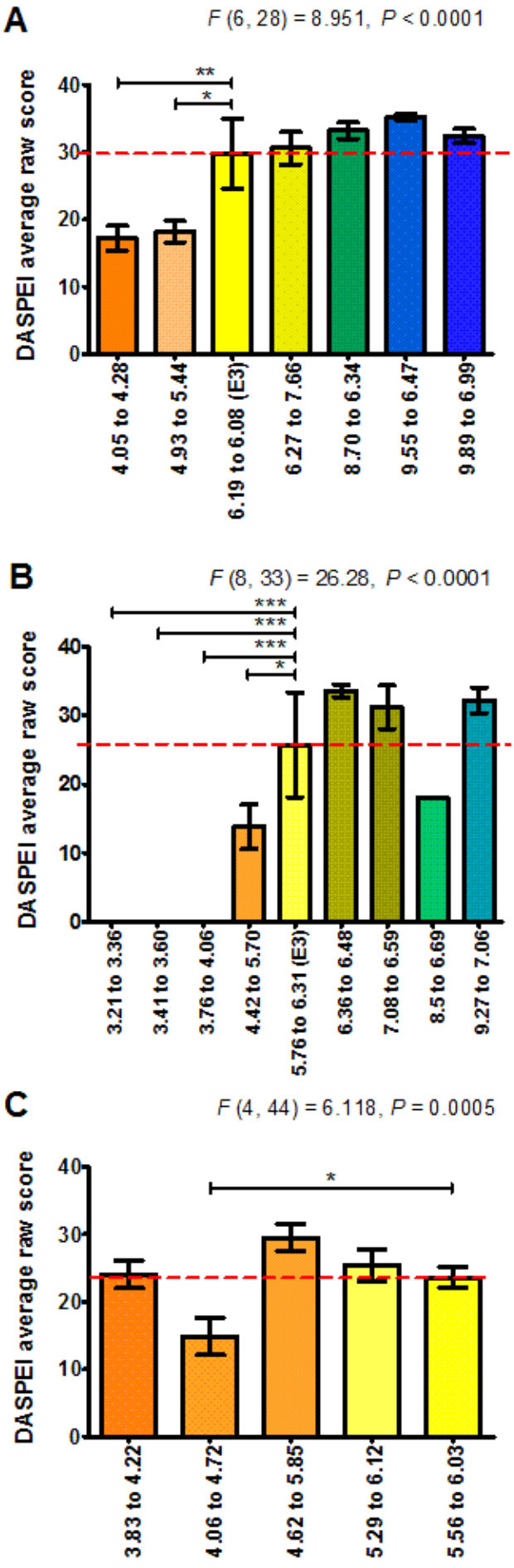



**Figure 3.11: pH alteration can affect DASPEI staining in control animals.** (A, B) In the initial two trials, lowering the pH appeared to have a negative effect on DASPEI fluorescence scores. (C) Follow-up testing in control animals at lower pH indicated that although pH alteration has an effect, there is a large amount of variability in DASPEI score. (A) and (B) *n*= 5 larvae per treatment group, (C) *n*= 10 larvae per treatment group. Test performed: One-way ANOVA followed by Dunnett's multiple comparison test.

### 3.13.3.2 Compound treated larvae

The effect of pH on DASPEI score was assessed using a selection of four weakly acidic compounds (Figure 3.12). These compounds were amoxicillin, melphalan, gentamicin and aspirin. These pH tests were performed in compound-treated larvae to ensure that the pH of these more acidic compounds did not have a damaging effect on the hair cells that was not due to ototoxicity.

Altering the pH of amoxicillin solution from 4.6 to 6.5 had no effect on DASPEI score in control or treated embryos ($F$ (1, 44) = 0.1059, $P$ = 0.7464). Treatment had no effect on DASPEI score at either pH, suggesting that amoxicillin did not cause toxicity to hair cells and that DASPEI staining was not affected by the weak acidity of the compound. This made amoxicillin a suitable negative control ($F$ (1, 44) = 0.9531, $P$ = 0.3343).

Altering the pH of melphalan solution from 4.5 to 6.2 had no effect on DASPEI score in either control or treated animals ($F$ (1, 36) = 0.2638, $P$ = 0.6106). Treatment with 0.4 mM melphalan had no effect on DASPEI score, suggesting it was not toxic to hair cells at either pH and was therefore a suitable negative control ($F$ (1, 36) = 3.13, $P$ = 0.0853).

Altering the pH of gentamicin from 4.8 to 6.4 had a significant effect overall ($F$ (1, 48) = 10.12, $P$ = 0.0026). Post-testing showed that changing the pH in control conditions had no significant effect on DASPEI score (Bonferroni test: $t$ = 1.670, $P$ = > 0.05). Altering pH to 6.4 may have significantly increased the toxicity of gentamicin to hair cells (Bonferroni test: $t$ = 2.889, $P$ = < 0.05) but the overall outcome remained the same; gentamicin treatment still significantly reduced DASPEI score in both pH conditions ($F$ (1, 48) = 618.5, $P$ = < 0.0001). Gentamicin thus appeared to be ototoxic in either condition.

Preliminary results showed that altering the pH of aspirin had a significant effect on DASPEI score, indicating that pH alteration was important in aspirin toxicity ($F$ (1, 36) = 1101, $P$ = < 0.0001). Overall, treatment with aspirin significantly affected DASPEI score, suggesting it was toxic to hair cells ($F$ (1, 36) = 1225, $P$ = < 0.0001). As expected for this experiment, there was a significant interaction between pH and treatment ($F$ (1, 36) = 1036, $P$ = < 0.0001). The interaction result showed that pH alteration did not have the same effect in control and

treated animals. Treatment with aspirin at the 0.4 mM level had significantly different effects under different pH conditions (Bonferroni test: $t$ = 46.22, $P$ = < 0.0001); pH alteration had no effect on DASPEI staining in control animals (Bonferroni test: $t$ = 0.7111, $P$ > 0.05). At the altered pH of 6.6, aspirin did not appear to affect DASPEI score compared to the control condition (2.6% change in DASPEI score). At the pH of 3.5, aspirin treatment decreased DASPEI score by 95% compared to control conditions. It was expected that the effects of aspirin treatment could be pH-dependent, as it is known that aspirin solubility is pH-dependent (less soluble in acidic conditions) and also that aspirin readily undergoes a neutralisation reaction in the presence of sodium hydroxide, converting it into the salt sodium acetylsalicylate plus water. The neutralisation reaction of aspirin may alter the toxicity of the compound to hair cells.

On balance, it was decided that alteration of the pH of compounds in solution was not necessary for the DASPEI assay.

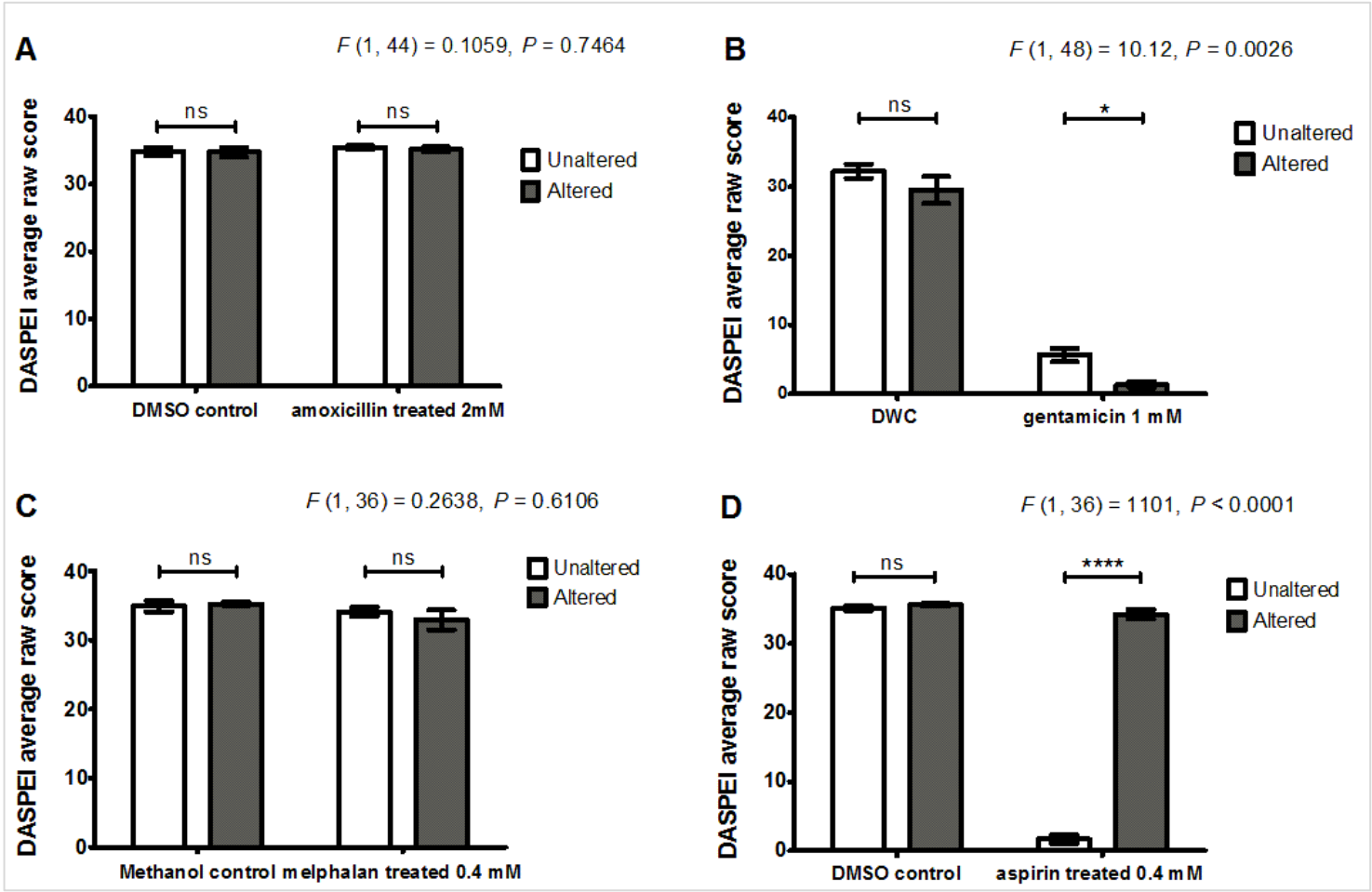



**Figure 3.12: The effects of pH on DASPEI staining in compound treated larvae.** The effects of acute treatment with amoxicillin and melphalan are independent of pH and are unaffected by pH alteration (A, C). (A) pH altered from 4.6 to 6.5. (C) pH altered from 4.5 to 6.2. (B) Treatment with 1 mM gentamicin is ototoxic in both non-altered (pH 4.8) and pH altered (pH 6.4) animals compared to matched controls. pH alteration significantly increases the damage caused by gentamicin treatment. (D) Treatment with 400 µM aspirin was ototoxic in non pH altered conditions (pH 3.5). Altering the pH of aspirin solution to match the pH of the DMSO control (pH 6.6) changed the outcome of the result, rendering aspirin ineffective at hair cell damage. All tests: Two-way ANOVA, followed by Bonferroni multiple comparison post-tests (minimum $n$ = 10 per treatment group).

### 3.13.4 Automation of DASPEI fluorescence intensity

The potential to automate the scoring of DASPEI fluorescence was investigated. It was hypothesised that the process of ototoxicity assessment might be shortened by automation.

#### 3.13.4.1 Automation of the DASPEI method using the Phenosight high throughput high-content screening system

Initially, I tested the Phenosight system to investigate the feasibility of high-speed automated scanning and quantification of DASPEI fluorescence as an assay for ototoxicity. Unfortunately, this system could not detect fluorescence, even in untreated DASPEI stained control animals at an exposure level of 40 ms (the highest exposure level on this system). Additionally, the orientation of the larvae in the 96-well format was poor.

#### 3.13.4.2 Automation of the DASPEI method using the Nikon AZ100 scanning microscope

Next, a system with a slower scanning time that was capable of longer exposures was tested. Using the Nikon AZ100 scanning microscope, it was possible to obtain images of sufficient quality to observe the neuromasts of control animals; this enabled the quantification of the effects of high-dose neomycin treatment. Quantification was carried out by measuring the fluorescence intensity along the lateral line and comparing the control versus treated groups. A one hour treatment with 100 µM neomycin significantly reduced the average difference in mean gray value compared to the dilution water control group (Figure 3.13; Mann Whitney U test, MWU statistic = 513, 1 *d.f.*, $P < 0.0001$). Although a significant difference was observed between the two groups, this system would be unsuitable for creating a concentration-response plot, as there were a number of difficulties with the image capture.

#### 3.13.4.3 Limitations of automation using the Nikon AZ100 scanning microscope

A number of issues were discovered upon inspection of the images obtained by scanning the 96-well plate. In particular, there were serious problems with orientation of the 5 dpf larvae. Crossover of the larvae, out of focus larvae, inappropriate orientation and curvature of the larvae all made quantification of the images far less accurate, leading to skewed data and some larvae being discounted altogether (see Figure 3.14 for examples). Gently spinning down the plates at low speed rpm on a plate centrifuge did not improve the orientation of the larvae.

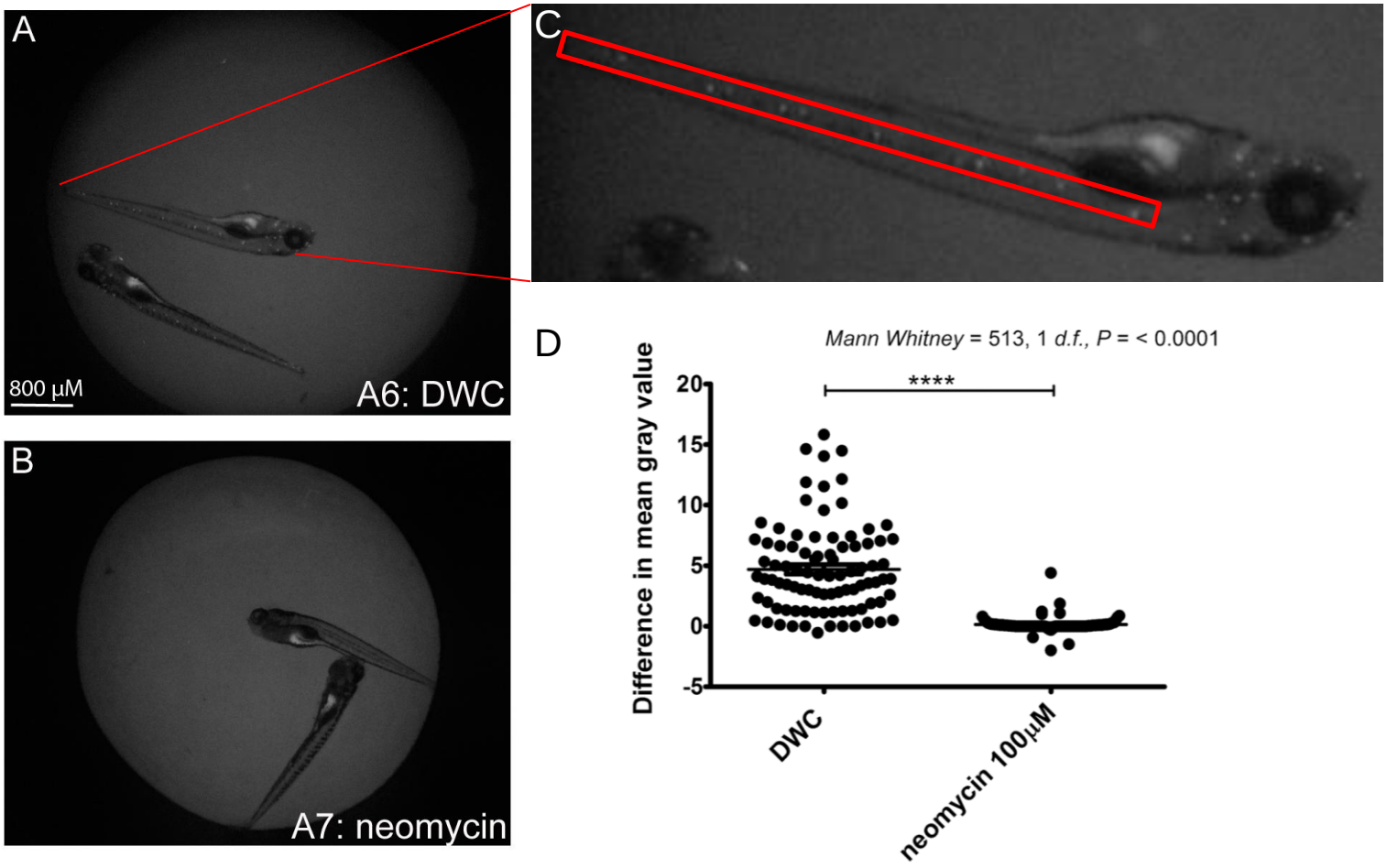



**Figure 3.13: Automation of the DASPEI fluorescence assay using the motorised Nikon AZ100 microscope.** (A, B) Examples of image capture using the Nikon scanning microscope. (A) Raw image obtained at 2x magnification in control animals from well A6 of a 96 well plate. (B) Raw image obtained at 2x magnification of DASPEI staining in neomycin treated animals. The image is of well A7 of a 96 well plate. (C) Enlarged image of a single larva from well A6, showing the region of interest (ROI) that was analysed. (D) Acute treatment with high-dose neomycin (100 µM) causes a significant reduction in fluorescence intensity/mean gray value (minimum $n$ = 89 per group over 1 trial , Mann Whitney U test, MWU statistic = 513, 1 *d.f.*, $P < 0.0001$).

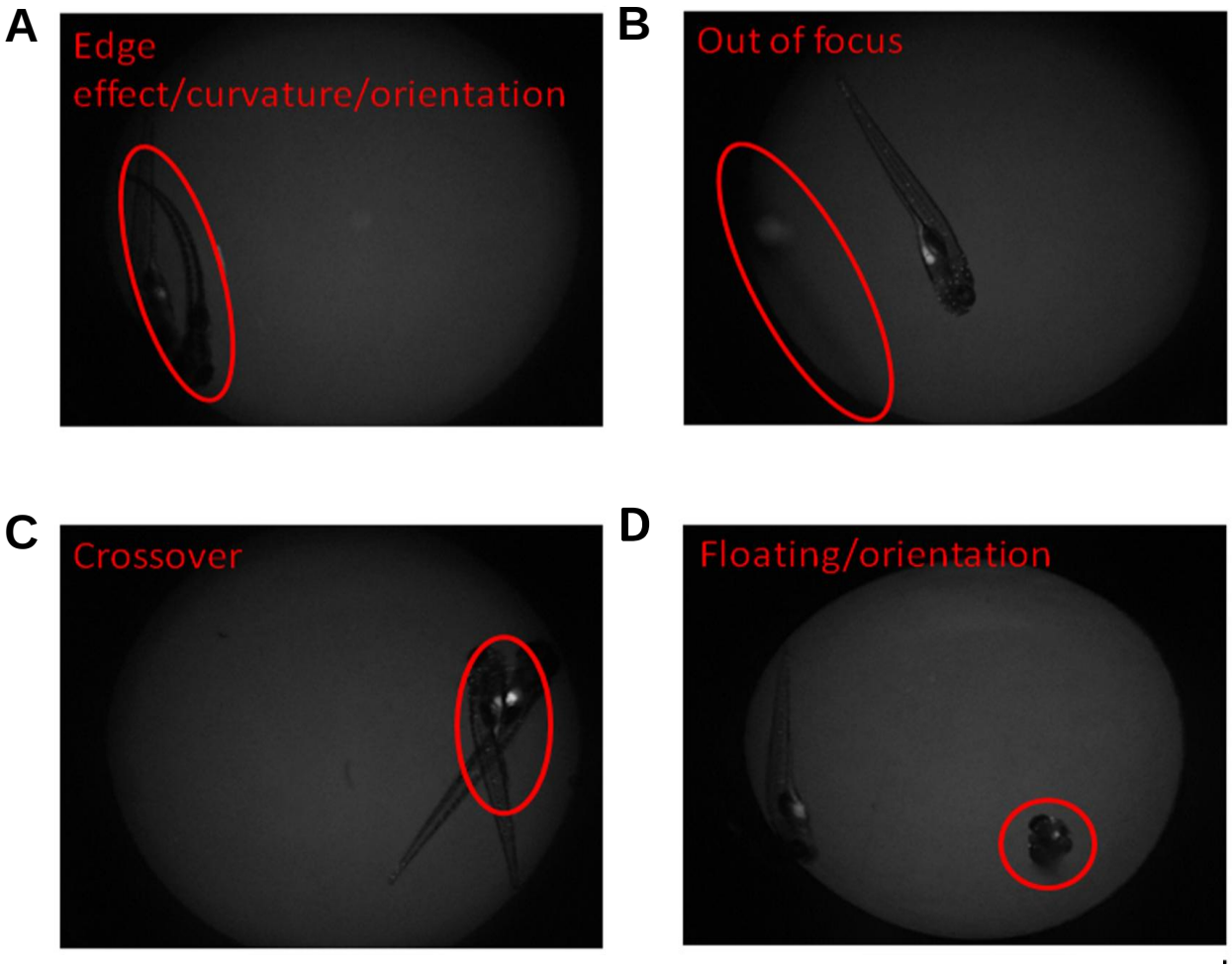



**Figure 3.14: Problems encountered with automated screening in the DASPEI assay.** The orientation and position of the larvae were important factors in measuring DASPEI fluorescence intensity. (A, B) Larvae were often out of the plane of focus or at the edge of the micro well. (C) Another common problem was crossover of the two larvae in the wells. (D) Inflation of the swim bladder at 5 dpf meant that a number of the larvae were floating and had altered orientation,. This made quantification of the fluorescence in the pLL difficult.

## 3.14 The effects of acute compound exposure on DASPEI staining score

### 3.14.1 Ototoxins

#### 3.14.1.1 Aminoglycosides

For all three aminoglycosides there was a concentration-dependent decrease in DASPEI score, indicative of hair cell damage or death (Figure 3.15). When compared at the concentrations of 50 and 100 µM, neomycin exerted the strongest effect on DASPEI staining at comparable concentrations, followed by gentamicin and streptomycin. For neomycin and streptomycin, a concentration of 100-300 µM was sufficient to eliminate the DASPEI stain ($P$ = > 0.05 from post-testing between 100, 200 and 300 µM over three repeats). The acute treatment given with gentamicin treatment was insufficient to completely abolish labelling. There was however, a concentration-dependent decrease in DASPEI score with the lowest observed effect concentration (LOEC) at 50 µM.

#### 3.14.1.2 Cisplatin

Acute treatment with cisplatin for 2 hours significantly decreased the average DASPEI score over three repeats (Kruskal-Wallis test, $H$ = 219, 7 *d.f.*, $P$ < 0.0001). The response was concentration-dependent, with the LOEC at 20 µM. According to post-testing, the average score was most reduced at a concentration of between 40 and 100 µM (Figure 3.15). Cisplatin treatments at higher concentrations for 1 hour were insufficient to cause the maximum damage and saturate the response (i.e. abolish the DASPEI stain), suggesting that the damage is time-dependent.

#### 3.14.1.3 Aspirin

Treatment with aspirin resulted in a concentration-dependent decrease in DASPEI fluorescence over three repeats (Kruskal-Wallis test, $H$ = 182.8, 5 *d.f.*, $P$ < 0.0001). The LOEC was 50 µM and DASPEI staining was completely abolished at concentrations above 300 µM (Figure 3.15).

#### 3.14.1.4 Copper sulphate

Unlike the clear concentration-response curves seen with the other human ototoxins, copper sulphate appeared to produce a threshold-type response (Figure 3.15). There was a sharp decrease in DASPEI average score at a

concentration of 0.5 µM. Immersion of larvae in concentrations below 0.5 µM did not significantly reduce DASPEI staining. Treatments above 0.5 µM caused a significant decrease in DASPEI staining, indicative of hair cell damage (Kruskal-Wallis test, $H$ = 405.2, 10 *d.f.*, $P$ < 0.0001). The response above 0.5 µM was, however, concentration dependent. Results from post-testing showed that DASPEI staining was completely abolished at concentrations above 0.7 µM (no significant difference between scores at 0.7 µM versus 1 µM (Dunn's test)).

#### 3.14.1.5 Furosemide

Furosemide failed to reduce DASPEI staining scores over three trials (Kruskal-Wallis test, $H$ = 3.841, 4 *d.f.*, $P$ = 0.4279, data not shown). For these reasons, furosemide was excluded from any further experiments.

### 3.14.2 Negative controls

#### 3.14.2.1 Amoxicillin, cefazolin and melphalan

Three of the four compounds selected as negative controls had no significant effect on hair cell labelling at the concentrations tested (Figure 3.15). This suggested that there was no detrimental effect of these compounds on hair cells.

#### 3.14.2.2 Gemcitabine

An initial treatment with the chemotherapeutic gemcitabine gave an unexpected positive result in the DASPEI assay (Figure 3.16). Contrary to expectations, this compound caused a significant reduction in DASPEI staining at levels of 50 µM and above (one-way ANOVA: $F$ (4, 21) = 197.3, $P$ = < 0.0001). Results from post-testing showed that DASPEI staining was completely abolished at concentrations above 250 µM. As a consequence of this result, gemcitabine was not used as a reliable negative control in subsequent assays. The possible reasons for this apparently positive effect in a previously assumed negative control are detailed in the Discussion part of this Chapter.

In all cases, nasal epithelium staining was unaffected by exposure to the ototoxins; this labelling served as an internal control for the reliability of the DASPEI staining method.

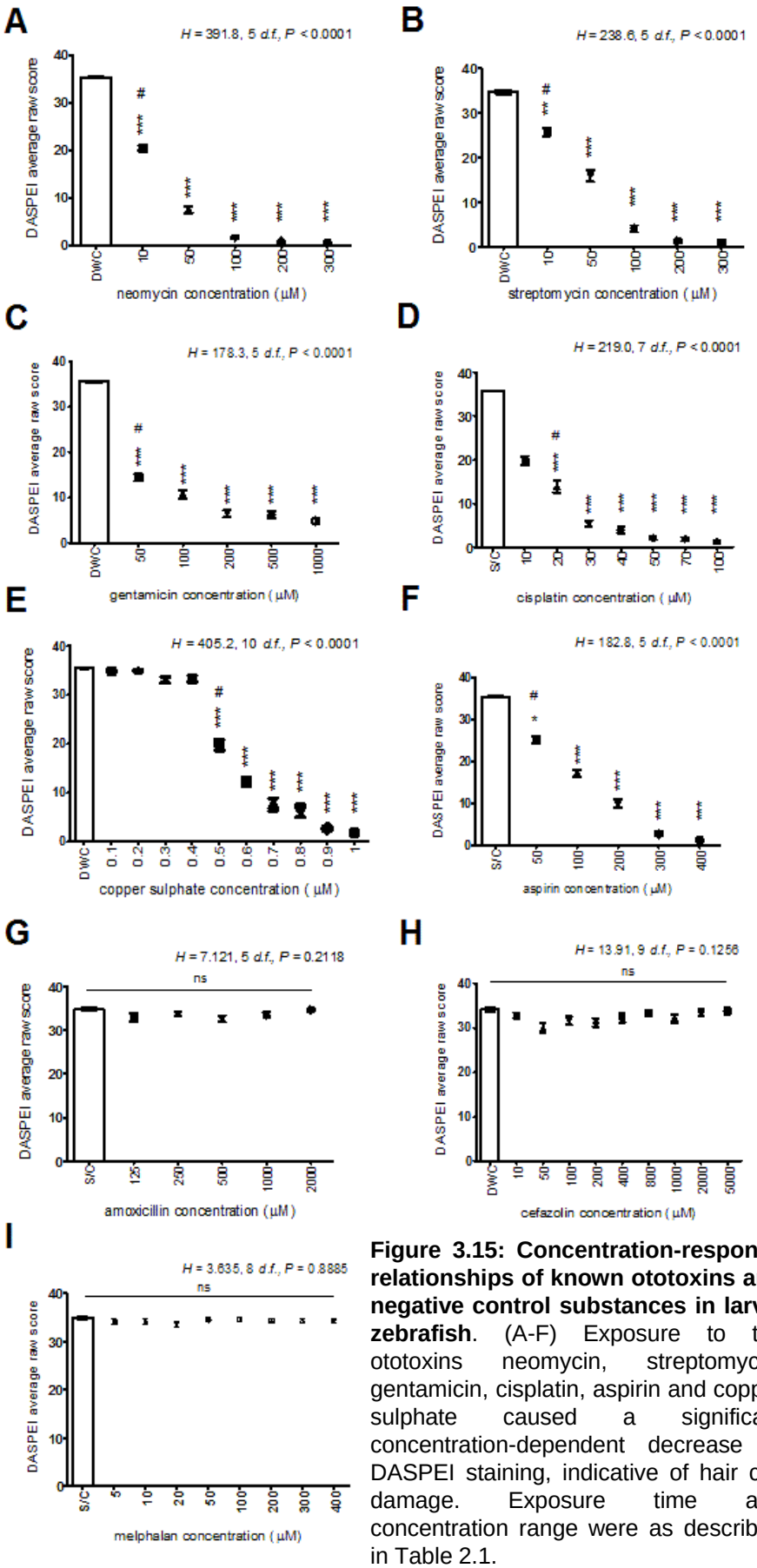
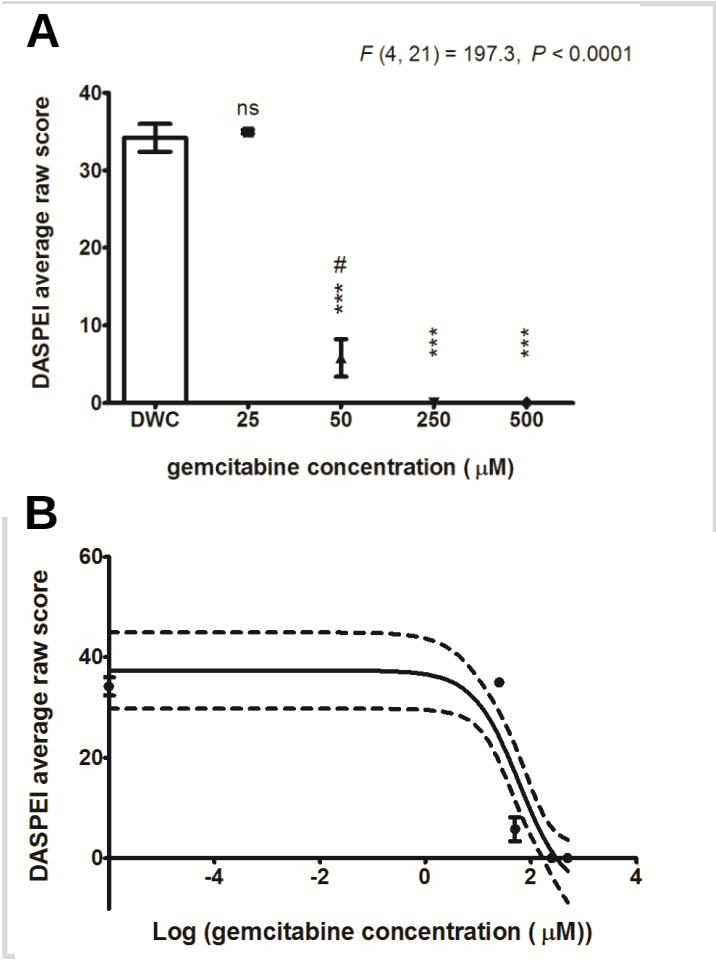
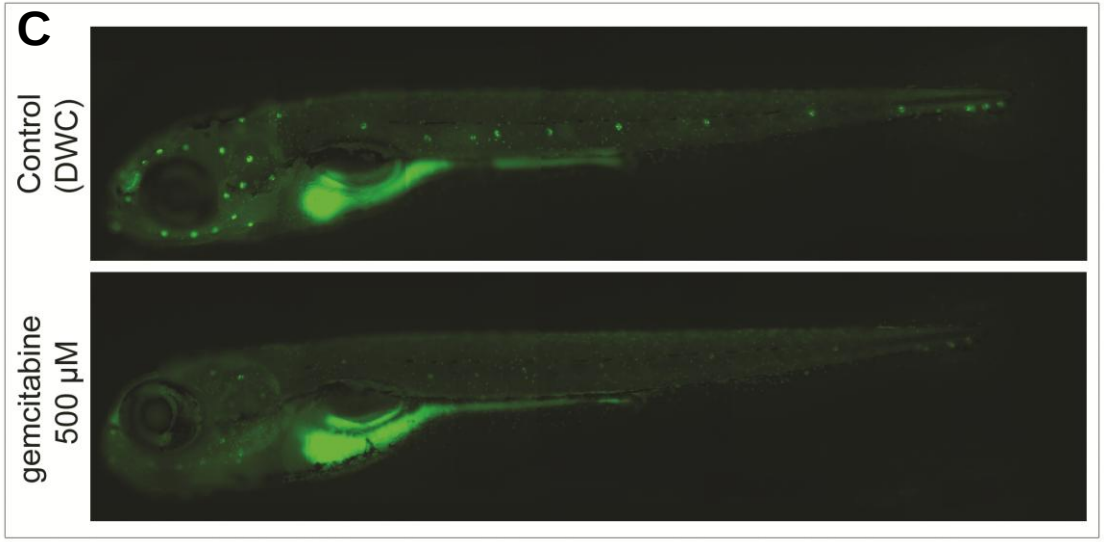



**Figure 3.15: Concentration-response relationships of known ototoxins and negative control substances in larval zebrafish**. (A-F) Exposure to the ototoxins neomycin, streptomycin, gentamicin, cisplatin, aspirin and copper sulphate caused a significant concentration-dependent decrease in DASPEI staining, indicative of hair cell damage. Exposure time and concentration range were as described in Table 2.1.

(G-I) Exposure to the negative controls amoxicillin, cefazolin and melphalan had no significant effect on DASPEI staining (Kruskal-Wallis test, followed by Dunn's multiple comparison test). Minimum of 29 fish per group (pooled from 3 or more experimental trials). Statistical significance compared with the control group is indicated by asterisks. *P < 0.05, **P < 0.01, ***P < 0.001, ns P > 0.05 (applies to all subsequent figures and tables). The first observed statistical effect is denoted by the hash (#) symbol.



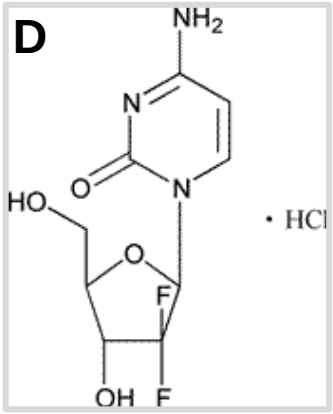




**Figure 3.16: The effects of gemcitabine on the pLL at 5dpf.** (A) Acute gemcitabine treatment induces an unexpected concentration-dependent decrease in DASPEI staining. (B) The IC50 of gemcitabine was estimated at by curve fitting to 59.25 µM. (C) Representative images of the damage induced by high-dose gemcitabine compared to DWC (*n* = 5 per group). (D) Chemical structure of gemcitabine hydrochloride.

## 3.15 Determining approximated $IC_{50}$ values for DASPEI fluorescence scoring data

The concentration of ototoxin required to reduce the DASPEI average raw score by half ($IC_{50}$) was approximated using curve fitting equations (Figure 3.17). In agreement with other studies, the order of potency for ototoxicity of the aminoglycosides could be ranked using the $IC_{50}$ value as: neomycin>gentamicin>streptomycin (Kotecha and Richardson, 1994; Owens et al., 2009; Wang et al., 1984). The $IC_{50}$ of cisplatin was most comparable with that of neomycin. Copper sulphate appeared to have the highest hair cell toxicity at the lowest treatment concentration. Aspirin required a much higher treatment concentration to elicit damage to the hair cells. These approximated $IC_{50}$ values were used in the startle, rheotaxis and avoidance assays as a mid-range treatment group to establish the sensitivity level of the assay and to investigate the comparative functional effect of hair cell damage induced by each compound.

## 3.16 The effects of acute compound exposure on GFP expression in the otic vesicle of larvae (*Tg(pou4f3::mGFP)s356t*)

In all cases, acute exposure to the test compounds did not result in a reduction in the GFP fluorescence of the otic vesicle of the larvae, or a change in the morphology of the maculae or cristae (Figures 3.7 and 3.18), even when there was a reduction in GFP in the pLL.

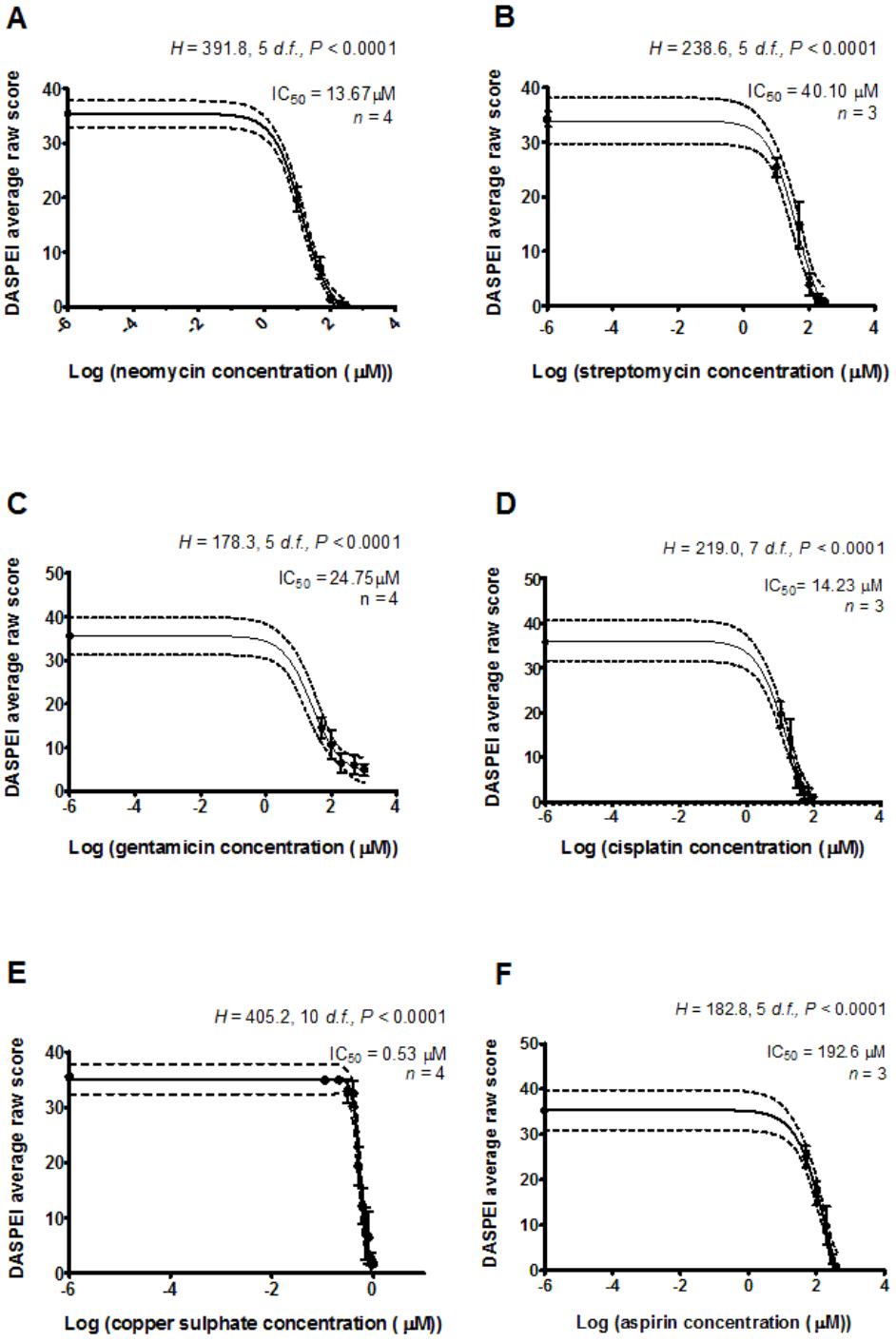



**Figure 3.17: Predicted $IC_{50}$ values for each of the ototoxins.** $IC_{50}$ values were approximated using either log(concentration of test compound) versus response (three parameters) or log(concentration of test compound) versus response - Variable slope (four parameters) curve-fitting equations from the original data shown in Figure 3.15. The $IC_{50}$ represents the dose required to reduce DASPEI average raw score by half and is used as a reference concentration in later chapters. Data were pooled from a minimum of 3 trials. Dotted lines represent the 95% confidence levels of each curve fitting.

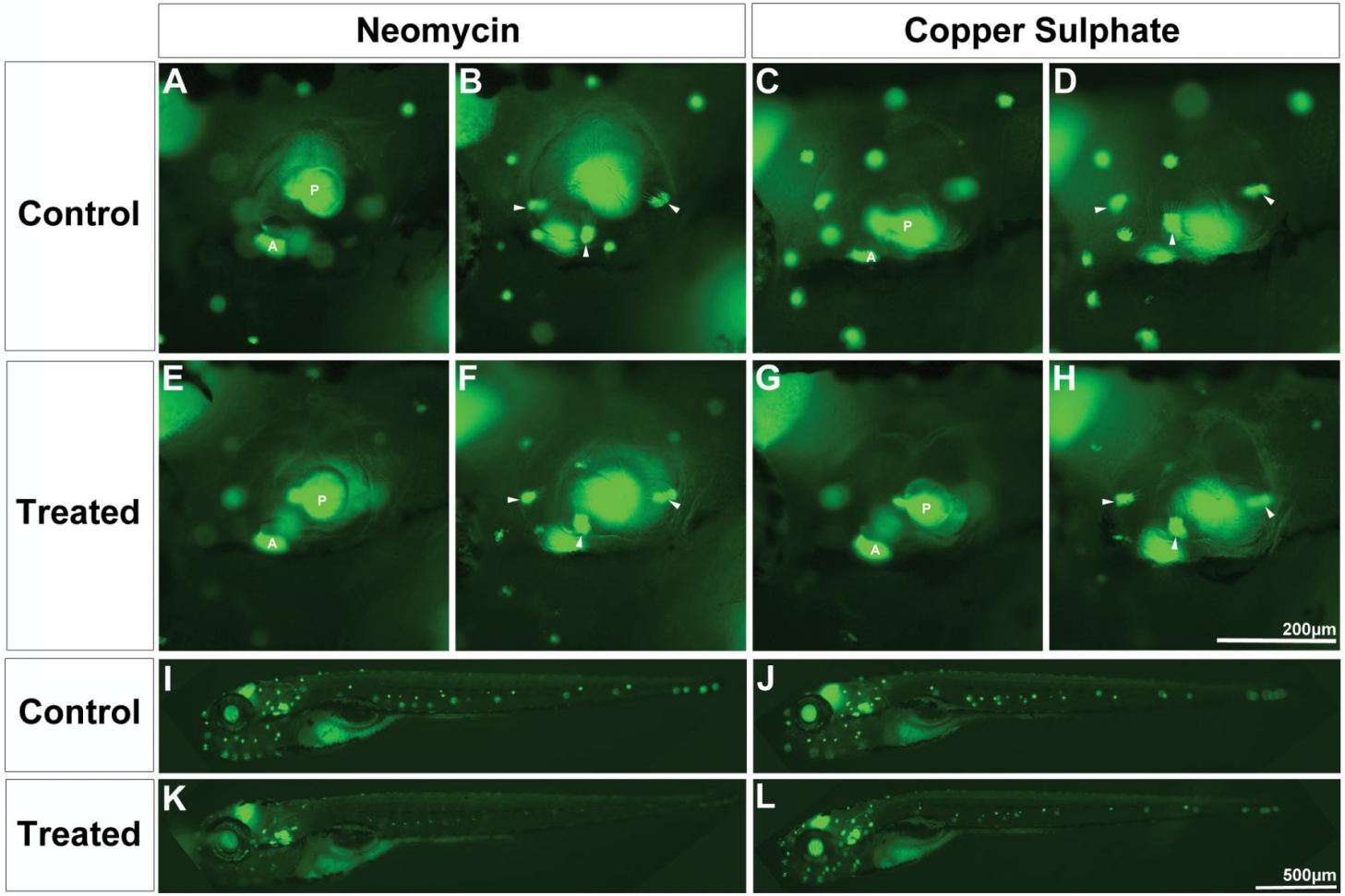



**Figure 3.18: The effects of copper sulphate and neomycin immersion on the ear and pLL of *Tg(pou4f3::mGFP)s356t* larvae.** Treatment with neomycin and copper sulphate (E-H) did not affect GFP expression in the hair cells of the inner ear when compared to control treatment (A-D). These images were taken at both the anterior and posterior focal planes in live animals. A, anterior macula; P, posterior macula; arrowheads indicate the cristae of the semicircular canals. Treatment with neomycin (K) but not copper sulphate (L) caused a strong decrease in GFP expression in hair cells of the pLL, when compared to control treatment (I, J). Images are representative of an *n* of 12 per treatment group.

## 3.17 The effect of ototoxin injection on the otic vesicle

In order to further assess the ototoxic potential of the compounds tested, the compounds neomycin, streptomycin and cisplatin were injected into the ears of *i193* larvae. Injection of 4 nL 100 mM neomycin was sufficient to reduce GFP expression dramatically in the posterior macula and in the anterior macula (to a lesser extent) when compared to control injected larvae (Figure 3.19, *n* = 15 from four trials for each treatment group). The cristae of the semicircular canals appeared unaffected by the ototoxins. Damage to the maculae was visible from 4.5 hours post-injection. Lower concentrations of neomycin (500 µM – 2 mM) did not damage the hair cells (data not shown). Preliminary data from streptomycin (*n* = 5 for each treatment group) and cisplatin (controls *n*= 8, cisplatin *n* = 7) injections showed the same pattern of preferential hair cell damage to the posterior macula (data not shown).

## Discussion

### 3.18 MTCs are useful indicators of compound toxicity

In this study, approximation of the maximum tolerated concentration (MTC) of each compound served as a qualitative indicator of compound toxicity; MTC determination not only acted as a starting point to determine appropriate test concentration ranges but also enabled the observation of the physiological responses of the whole animal to toxic insult. Overall, the results obtained from initial MTC experiments fit with those from other studies. Specifically, it has been shown that cisplatin was toxic at concentrations above 1 mM. Only one published study has exceeded this concentration at the same larval stage. Ou and colleagues immersed larval zebrafish in 1.5 mM of cisplatin for 4 hours (Ou et al., 2007). However, in that study, the cisplatin was dissolved in E3 directly instead of DMSO and the larvae were fed at 4 dpf (potentially making them hardier). Overall, reported concentrations used by others range from 50-1000 µM which is within the non-toxic range used in this study. The MTC assessments of neomycin presented here also fit with the published literature. The highest reported concentration used was 1 mM for 4 hours, although neomycin was typically used at concentrations between 100 and 400 µM. It is widely accepted that 200 µM is sufficient to induce the maximum hair cell damage (Ou et al., 2009). This is the concentration within the non-toxic range established in my study (Buck et al., 2012).

### 3.19 MTC versus MTD in assessing toxicity

There is a clear requirement to test for lethality and multiple-organ damage when assessing specific sub-lethal pharmacological toxicities in larval zebrafish (Hutchinson et al., 2009). This simple assessment ensures that any effects seen within the system of interest, the lateral line in this case, are organ specific. Although the MTC appears to be a reasonably accurate reflection of the concentration eliciting >20% lethality, the maximum tolerated *dose* (MTD) cannot be easily ascertained. For both the DASPEI and MTC assays, it would have been ideal to ascertain how the MTC score corresponded with the internal concentration (i.e. dose) of compounds within the whole animal. Due to the individual chemical properties of the compounds that were tested, it was not

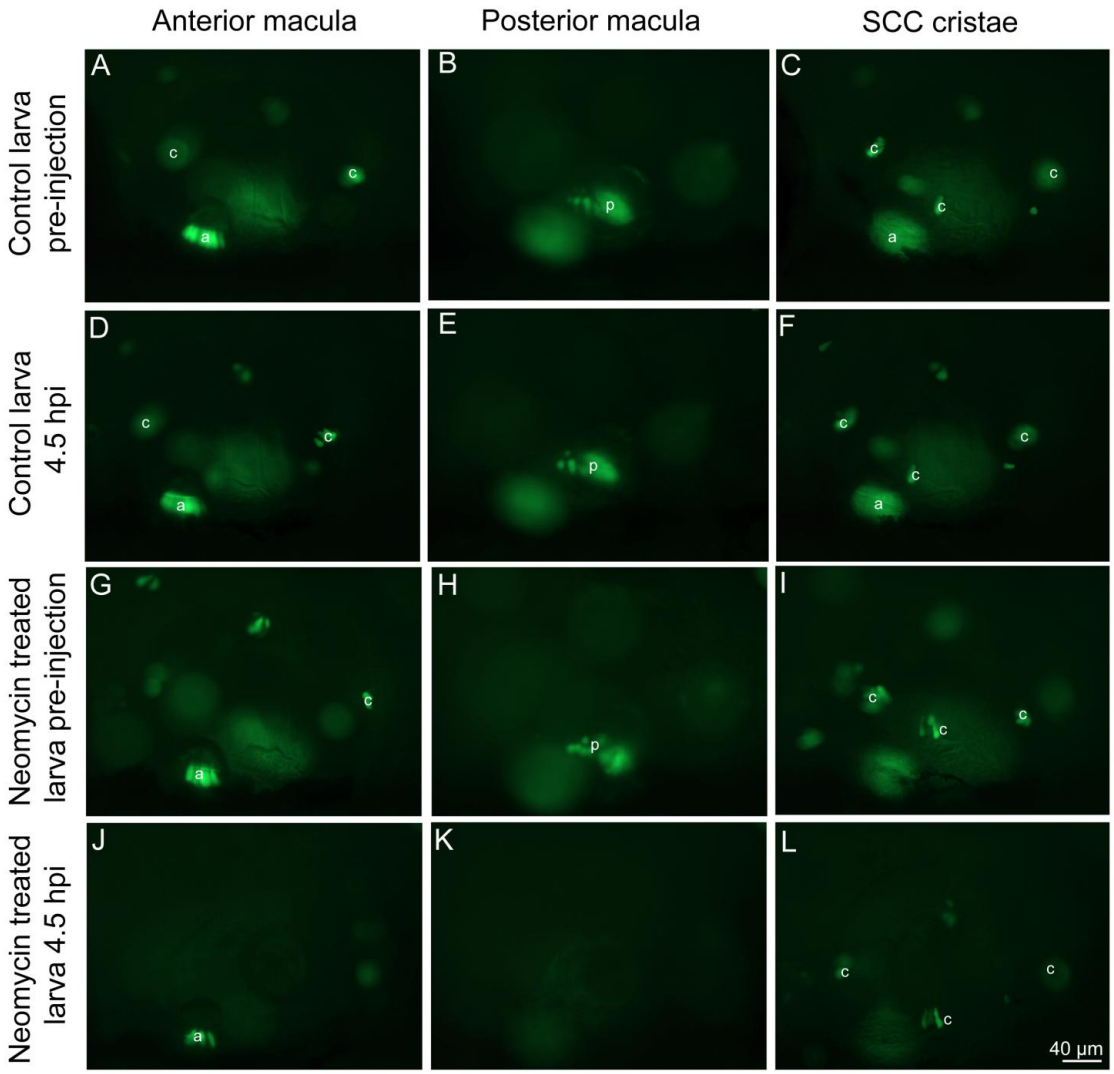



**Figure 3.19: The effects of ototoxin injection on the sensory patches of the zebrafish ear at 4 dpf**. Panels show the effects of injecting larval ears with either a 10% phenol red dilution water control (A-F) or with 4 nL of 100 mM neomycin (G-L) and re-imaging 4.5 hours post-injection. Injection of neomycin damages the anterior macula and posterior macula within 4.5 hours (J, K) with no observable damage to the cristae (L). *A*, anterior macula; *P*, posterior macula; *C*, cristae of the semicircular canals. Images are representative of n = 15 per treatment group.

possible to investigate uptake using a well established liquid chromatography mass spectrometry (LC/MS) method.

Although bioanalysis was not possible during the current study, previous unrelated studies have shown that in general, antibiotic compounds (e.g. cefazolin, tobramycin and amoxicillin for example) show relatively poor uptake into the larva at 7 dpf (M. J. Winter, personal communication). Despite this, it is worth reiterating that systemic exposure is not necessarily required to induce hair cell damage, as the neuromasts are directly exposed to the compound in solution.

## 3.20 Factors such as pH and duration affect the strength of DASPEI staining

It was found that factors such as the duration of staining and pH of exposure solutions could affect the outcome of DASPEI staining, whereas the anaesthetic MS222 had no significant effect.

It was expected that the duration of exposure to the dye would affect the strength of the stain as DASPEI is known to be taken up slowly into the cells as a function of membrane potential. It was, therefore, necessary to determine the optimal duration of immersion in the untreated larvae to obtain the most robust results. This was established to be twenty minutes in 5 dpf wild-type larvae.

Although pH had some effects on DASPEI staining, it was decided that the pH of compounds would not be altered to within set limits before testing for ototoxic effects. The reason for this decision was that in most cases where pH was out of normal range (amoxicillin, melphalan and gentamicin) altering the pH did not alter the outcome of the test (i.e. whether the compound was identified as ototoxic). In only one instance, altering the pH affected the outcome of the experiment; the known human hair cell toxin aspirin was no longer identified as ototoxic at pH > 6.5. This was most likely due to the dissociation of the compound. Aspirin readily undergoes a neutralisation reaction in the presence of sodium hydroxide, converting it into the salt sodium acetylsalicylate and water, thus rendering it inactive.

For the purposes of this study, as long as non-specific toxicity was controlled for, it was preferential not to mask the effect of a potential ototoxin by changing its chemical composition through the alteration of pH.

## 3.21 The automation of DASPEI scoring was limited by larval orientation

The feasibility of automation of the image capture, processing and scoring for the DASPEI assay was investigated. I hoped to develop an automated DASPEI screen which was faster than scoring manually and could produce a more sophisticated quantification (providing continuous rather than discrete quantitative data).

The Phenosight system is a high-speed plate reader for fluorescence analysis. The system was tested using control and neomycin treated larvae stained using DASPEI. Unfortunately, the plate reader used was unable to detect the staining in the control larvae even at the maximum possible exposure level.

Next, I tested the Nikon plate scanning system. This system was slower to scan the microplate but produced much better images using a 1 second exposure and EDF (extended depth of focus) imaging. Despite the improved image capture, there were still a number of disadvantages associated with this semi-automated method for DASPEI assessment compared to manual assessment.

The key issue identified was that the orientation of the larvae in the wells of the microplate was not ideal for viewing the neuromasts. The optimal position was with the larva lying in a lateral position, so that all neuromasts on one side could be imaged. Often, larvae were in alternative positions such as those displayed previously (Figure 3.14). This made the readings of fluorescence intensity inaccurate. Unlike in younger embryos, where larvae are more likely to position in the centre of the well and sink to the bottom of the plate, larvae at 5 dpf float due to the inflation of the swimbladder; this makes focussing of images difficult, even with multiple z-stacking and EDF processing. Deflating the swimbladders of the animals would be too impractical for an automated or even medium throughput approach.

There were a few additional problems with automation on the Nikon system. Firstly, the total time taken to load the plates with larvae and to analyse the images manually was greater than the time taken to score the larvae by eye. Secondly, fewer data were sampled by scanning the larvae, as the measure of fluorescence was often only for one side. This differed from the manual scoring, where both sides of the fish were scored in all cases.

One way to correct for the imperfect orientation of the larvae, to accelerate loading of the animals into the plates and to improve the amount of data obtained (i.e. imaging both sides of the subject and fitting to the specified region of interest) would be to employ an advanced capillary imaging system (Chang et al., 2012; Pardo-Martin et al., 2010). The vertebrate automated screening technology (VAST) method is a recently developed technique for high-throughput confocal imaging. This system loads and unloads larvae automatically from a 96-well mesh plate using fluid handling systems. VAST is capable of processing multiple larvae simultaneously at loading, imaging, and unloading steps. Larvae are dispensed into a capillary which is rotated by two stepper motors, enabling 360 degree imaging of larvae. The VAST system is not currently available at the University of Sheffield or Brixham Environmental Laboratory. An alternative way to address the problem of orientation would be to use u-shaped black microplates to manipulate larvae into the central position in the well, as used by Walker and colleagues in their high-throughput screen (HTS; Walker et al., 2012).

A template for the region of interest (ROI) was created in the software to ensure that an equal area was measured for each of the direct comparisons. This proved inflexible when larvae were orientated in the wrong plane. A way to improve this system might be to assess the neuromasts individually rather than drawing a rectangle along the entire pLL and by designing an algorithm to recognise the shape of each neuromast, such as that used in the chemically induced inflammation (ChIn) assay (d'Alençon et al., 2010). Cognition Network Technology (CNT) is an object-oriented method of analysis that was developed to inspect satellite images. The technology has more recently been developed to recognise components of the whole zebrafish embryo such as yolk and head and to successfully quantify the brightness and area of GFP expression. It is

compatible with automated scanning in the 96-well format and has thus far been used to investigate the modulation of the FGF/RAS/MAPK pathway in embryos and to quantify intersegmental blood vessel development in embryos treated with small molecule inhibitors of anigiogenesis (Vogt et al., 2009; Vogt et al., 2010). CNT has the potential to be adapted to study neuromasts. A final alternative would be to use Automated Reporter Quantification in vivo (ARQiv). ARQiv uses a microplate reader with modified detection functions. Imaging using ARQiv could be advantageous in future assays of this kind, as it allows changes in fluorescent reporters to be accurately monitored over time. So far, this method has been used to compare the variability of reporter expression in transgenic lines, to investigate the modulation of the Notch pathway and to quantify increases in reactive oxygen species (ROS) production (Walker et al., 2012).

Currently, the best method of evaluating DASPEI staining in the animals is by manual manipulation and scoring by eye. This method provides fast and reliable results. In future, it may be beneficial to optimise the Nikon system for scanning, or to implement a high speed VAST-type method in assaying for hair cell damage. There is much scope to develop the automation of the assay further as numerous alternative technologies have been applied to HTS of the zebrafish for other endpoints.

## 3.22 The effect of ototoxins on the pLL

Many compounds are considered to be toxic to human and mammalian hair cells (Guthrie, 2008; Rybak and Ramkumar, 2007; Rybak et al., 2007). Larvae were exposed to a small collection of known ototoxins (or to negative controls with no reported effect in the ear) in order to investigate the sensitivity of zebrafish hair cells to these compounds.

A fluorescence scoring system was employed in order to quantify hair cell damage in the pLL. For scoring, larvae were immersed in the styryl dye DASPEI. This dye is readily taken up by the sensory hair cells of the neuromasts and by cells of the nasal epithelium. The level of DASPEI staining observed correlates to the number of functioning hair cells present in the

neuromast; this has previously been shown using methods such as phalloidin and anti-acetylated tubulin antibody labelling (Harris et al., 2003).

The aminoglycoside antibiotics, cisplatin and copper sulphate all induced a concentration-dependent loss of DASPEI staining in the pLL, indicative of hair cell damage. Moreover, exposure to ototoxins reduced FM1-43FX staining and GFP labelling in hair cells, further indicating hair cell damage and loss. This is in agreement with previously published data in zebrafish (Chiu et al., 2008; Coffin et al., 2010; Harris et al., 2003; Hernandez et al., 2006; Ou et al., 2007; Ou et al., 2010; Owens et al., 2007; Owens et al., 2009; Ton and Parng, 2005; Van Trump et al., 2010). In addition, the salicylate aspirin, a reported reversible ototoxin in humans, was tested and it was found that similar concentration-dependent hair cell damage occurred with DASPEI and GFP but not FM1-43FX labelling. This result suggested that although the mitochondria encounter the damage, some aspirin-treated hair cells may maintain mechanotransductive activity and are, therefore, still functioning.

One known human ototoxin, furosemide, failed to induce significant damage to the hair cells of the pLL in zebrafish. This result was somewhat expected, as furosemide was also not detected as a hair cell toxin in a large compound screen for ototoxins (Chiu et al., 2008).

Further testing of the positive control neomycin provided evidence that apoptotic cell death could occur in response to treatment. Nuclear condensation and fragmentation was observed in the live fish following neomycin treatment and staining with DAPI. In support of this, there was also an increase in TUNEL staining in neomycin-treated animals at the highest concentration tested. This is in agreement with other studies (e.g. Williams and Holder, 2000). Although it appears that most of the cell death caused by the aminoglycosides is apoptotic, necrotic death cannot be ruled out (Grasl-Kraupp et al., 1995). In future, it would be valuable to test the effects of the ototoxins in a way which allows measurement of the levels of cell death. In this respect, the availability of the new transgenic line secA5-YFP, which acts as a marker of apoptosis, could make it possible to visualise apoptotic cell death in real time (van Ham et al., 2010).

Interestingly, cisplatin treatments at higher concentrations for one hour were insufficient to cause the maximum damage to the hair cells; a two hour exposure period was necessary to observe the full effects of this ototoxin. This slower time course for damage has also been observed by others (Ou et al., 2007), and suggests that the aminoglycosides and cisplatin may have distinct mechanisms of hair cell damage. There are a number of possible reasons for this. For example, the uptake and clearance mechanisms in the cell may differ between the two drug classes. Also, it is likely that there are some differences in the mechanisms of hair cell death once inside the cells. For example, neomycin and cisplatin may act through different apoptotic pathways; blocking Jun kinase with d-JNKI-1 attenuates neomycin- but not cisplatin-mediated hair cell death (Eshraghi et al., 2007; Wang et al., 2004).

A small group of negative control compounds with similar therapeutic effect in humans to the ototoxins, but no documented ototoxic side effects were also examined. These included the antibiotics amoxicillin and cefazolin and two chemotherapeutics melphalan and gemcitabine. Cefazolin and amoxicillin have not previously been investigated for their ototoxic effects in the zebrafish. The chemotherapeutics melphalan and gemcitabine were shown in a recent zebrafish screen to have no hair cell toxicity (Hirose et al., 2011). In my assay, amoxicillin, cefazolin and melphalan had no significant effect on hair cell staining, supporting evidence that they exert no ototoxic effects. In contrast to the findings of the published screen (Hirose et al., 2011), gemcitabine exposure resulted in a significant reduction in hair cell labelling, thus scoring as positive in the current assay. One possible explanation for this discrepancy is that DASPEI labels mitochondria, whereas Hirose and colleagues used the nuclear dye YOPRO-1, which could be less sensitive to more subtle cellular alterations caused by treatment. One rationalization for the lack of reported ototoxicity of gemcitabine in humans is that patients treated with gemcitabine are commonly treated with gemcitabine-cisplatin adjuvant therapy (Joint Formulary Committee, 2011; Lee et al., 2004b). The effects of cisplatin may therefore mask any additional ototoxic effect of the gemcitabine in patients. Additionally, the concentration of gemcitabine used in my experiments is likely to be higher than the effective concentration used in humans, due to the direct exposure of the

neuromasts to the compound. The human ear is less likely to encounter such high levels.

Previous work has described the role of the external ionic environment in hair cell death and protection in the zebrafish lateral line. It is known that increasing calcium or magnesium in external bathing solutions can protect hair cells from acute neomycin and gentamicin damage by blocking their cellular uptake (Coffin et al., 2009; Kaus, 1992). To control for the effect of cations in this study, all larvae were raised and tested in embryo medium with identical composition at both the University of Sheffield and at AstraZeneca, Brixham.

It was important when designing this assay to ensure that the data were robust. It is noteworthy that, over repeated trials, the effects of the compounds were consistent, even when tested across two different zebrafish strains. All compounds were tested in WIK larvae once to ensure that the outcomes matched with the data obtained for the AB animals (data not shown). Across the strains, the ototoxicity was still observed. This is a positive indicator of the strength of the assay.

One area for further investigation would be to examine the effect of the ototoxins on support cells of the neuromast in detail. Although I did not investigate the effect of the ototoxins on support cells, previous studies have been conducted to investigate this. Specifically, there is evidence that high concentrations of copper sulphate can damage the structure of the support cells (Olivari et al., 2008). Studies into regeneration in the lateral line suggest that neomycin may spare support cells at the concentrations used here: hair cells can regenerate following neomycin treatment and this regeneration is thought to involve support cells (Harris et al., 2003). The focus of the current study was specifically on the hair cells of the lateral line, as these are the first cells to be damaged, making for a more sensitive assay of ototoxicity.

In order to fully validate the DASPEI assay as a pre-clinical indicator of ototoxicity, it would be necessary to carry out a blinded screen using a much larger selection of compounds. This would act as a more detailed indicator of the sensitivity and specificity of the assay. These experiments extend beyond the remit of the study but are an area for future development in order to validate

this method as a preclinical assay for the assessment of ototoxicity in new drugs.

## 3.23 The effect of ototoxins in the inner ear

To investigate the ototoxins in more detail, the outcome of compound exposure in the ear was tested. The aim was to ascertain whether hair cell damage could be induced in the inner ear, as in humans, other mammals and birds. Additionally, these experiments served to reveal whether there would be preferential damage to one of the sensory patches, which may relate to the functional damage in humans. Finally, it was hoped to use these data to compare the effects of the ototoxins between the zebrafish and other vertebrate models and to assess the suitability of the zebrafish as a surrogate model to predict human ototoxicity.

Initially, the aim was to induce hair cell damage in the otic vesicles of the fish by immersion of the larvae in solutions containing ototoxins. However, it was found that neither acute nor chronic treatment with ototoxins affected the levels of GFP expression or the morphology of inner ear hair cells. It has been proposed that acute exposure of larvae to ototoxins causes damage specific to the lateral line that does not affect the ear (Blaxter and Fuiman,1989; Matsuura et al., 1971). This is likely to be because ototoxins dissolved in the medium in which the larvae are swimming are less able to access the ear compared to the easily accessible lateral line.

To answer the question of whether ototoxins could damage hair cells of the inner ear, compounds were injected postero-laterally into the otic vesicles of larvae at 4 dpf using the *i193* transgenic line. As in humans, hair cells of the ear were susceptible to insult with neomycin, streptomycin and cisplatin in zebrafish larvae. The posterior (saccular) macula (whose role is thought to be primarily auditory; Fay and Popper, 1999) was severely affected. The anterior (utricular) macula (thought to have a primarily vestibular role; Fay and Popper, 1999) was damaged and cristae of the semicircular canals were also affected, but to a lesser extent. The cristae retained their GFP expression and their morphology in the main. In humans and other vertebrate models treated with neomycin, the damage occurs mainly in the auditory OHCs at the basal turn of the cochlea.

These results seemed to match with what is observed in other model systems at least in part. In addition, I tested the effect of streptomycin injection on the inner ear. The hypothesis was that injection of a more vestibulotoxic compound may preferentially damage the utricular macula and/or cristae. Interestingly, streptomycin injection, as with neomycin injection, preferentially damaged the saccular macula. An explanation for the susceptibility of the saccular macula to ototoxic insult may be that it is better developed than the other patches at 4 dpf and has a higher compound uptake overall. This may be because the cells are more mature and have more active uptake. When tested, it appeared that hair cells in all of the sensory patches at 4 dpf were mechanotransductively active, as they were all capable of FM1-43FX uptake. To my knowledge, nothing in the literature concerning the development of the sensory maculae suggests that one macula develops faster than the other.

## 3.24 Concluding remarks

The DASPEI assay in larval zebrafish appears to offer a robust and sensitive method for the detection of potential ototoxins. I have shown that the hair cells of the zebrafish lateral line and inner ear can be damaged by exposure to known human ototoxins. These data concur not only with the literature from zebrafish but also from higher vertebrates and humans. Taken together these data support the use of the zebrafish as an early stage pre-clinical indicator of drug-induced ototoxicity.

Although histological changes following exposure to selected human ototoxins were demonstrated it was necessary to show that this cellular damage translated into a functional consequence, impaired auditory function. In other words, it was important to demonstrate that zebrafish larvae experience the decrease in auditory function seen in higher vertebrates and humans as a result of ototoxic insult. Assays to measure the functional consequences of hair cell damage are described in the following Chapters.

# Chapter 4 Optimisation of the startle assay

## Introduction

### 4.1 Aim

The aim of the work described in this chapter was to develop and optimise a semi-automated method for assessing the high-speed auditory evoked response (or startle) in 5 dpf larval zebrafish. Once optimised, the goal was to use this method, in conjunction with other simpler tests for reflex behaviours, in order to determine the functional consequences of the hair cell damage seen in the histological DASPEI assay for individual compounds.

### 4.2 The larval startle response

As discussed in Chapter 1, zebrafish are hearing specialists. They use their *acoustico-lateralis* system to facilitate a number of essential behaviours including feeding, schooling and predator avoidance (Dijkgraaf, 1963; Ghysen and Dambly-Chaudière, 2004; Kaus, 1987). Vibrations through the water and water flow are detected using the specialised mechanosensory hair cells in the neuromasts, which elicit a variety of reflex behaviours (McHenry et al., 2008; Zeddies and Fay, 2005).

A key locomotor behaviour exhibited by larval zebrafish is the startle response. The acoustically evoked startle response is conserved in most vertebrate species including humans (Landis and Hunt, 1939), but is especially well characterised in fish and conserved in species such as *Carassius auratus* (goldfish) and *Lepomis macrochirus* (bluegill) (Neumeister et al., 2008; Zottoli, 1977). Startle is crucial in escaping the strike of a predator; it can be elicited by water flow, touch and visual cues (Colwill and Creton, 2011; Issa et al., 2011; Kohashi and Oda, 2008; McHenry et al., 2009). Zebrafish larvae are capable of performing this complex, high-speed escape response from five days post fertilisation onwards, correlating with the mechanotransductive maturity of the hair cells and swim bladder inflation (Bang et al., 2002; Murakami et al., 2003; Santos et al., 2006). It is suggested that the sensitivity of larvae to respond with a start to vibratory cues remains unaltered after this time, making this the ideal age to study functional changes in response to compounds that cause hair cell damage (Zeddies and Fay, 2005).

The startle response is characterised by an initial bend involving the whole body (with an estimated angle of 90-220º) occurring in the first 14 ms after stimulation, followed by a strong counter-flexion and subsequent smaller bends. In total a startle lasts for approximately 40 ms.

Like in goldfish, the zebrafish escape response is mediated by the M-series reticulospinal neurons (Foreman and Eaton, 1993; Kimmel et al., 1980; Weiss et al., 2006). Ablation studies and in vivo calcium imaging have shown that Mauthner, MiD2cm and MiD3cm neurons integrate convergent sensory input and send motor commands to initiate the reflex (Gahtan et al., 2002; Issa et al., 2011; Kimmel et al., 1974; Kimmel et al., 1980; Liu and Fetcho, 1999; Liu et al., 2003; Sillar, 2009). The action potential created by the reticulospinal neuron stimulates the contralateral trunk muscle to bend the entire body. The C-start can be described as short or long latency (SLC and LLC respectively), with the SLC being the stronger Mauthner cell dependent response (Burgess and Granato, 2007).

It is noteworthy that a less well characterised S-start is also described in zebrafish. This escape response is mainly elicited through stimulation of the tail and has a similar onset and conduction velocity to an SLC. The S shape of the response is made by the contraction of rostral axial muscle on one side of the body and caudal axial muscle on the opposite side. Motor control of the S-start is poorly understood but responses may be elicited by an alternative reticulospinal circuit, a combination of reticulospinal and local circuits, or rhythmic swimming circuits (Liu et al., 2012). The lateral line is a key mediator of larval startle responses, but the ear is also proposed to contribute to the response; this will be discussed in more detail in the following chapter.

Previous studies on the startle response in zebrafish have mostly focussed on methods of characterising and measuring the response (high-speed kinematics, video based frame subtraction), age of onset of the reflex and how the response changes during development (Budick and O'Malley, 2000; Fontaine et al., 2008; Gahtan et al., 2002; Kimmel et al., 1974; Kimmel et al., 1980; Liu et al., 2003; Muller and van Leeuwen, 2004; Sillar, 2009; Zeddies and Fay, 2005). In addition, acoustic stimulation and startle measurement has been used in other

contexts such as measuring the effects of compounds on associative learning (Best et al., 2008; Roberts et al., 2011; Wolman et al., 2011) and Pre-pulse Inhibition (or PPI) (Burgess and Granato, 2007). Only one study has been carried out to determine the effects of a single ototoxin on startle response and this investigation was based solely on touch-evoked startle (McHenry et al., 2009), not auditory evoked startle.

## 4.3 Measuring sound waves

One key aspect of this work was ensuring the quality and purity of the sound waves that were generated in order to elicit the startle response.

### 4.3.1 Important properties of sound waves

Sound is a mechanical vibration which travels in longitudinal (or compressed) waves in air and water. A simple sound wave has a number of key properties including: frequency, amplitude, direction and sound pressure.

#### 4.3.1.1 Frequency

Frequency is defined as a number of cycles of a wave per unit time. The Hertz (Hz) is the unit of frequency and is the number of cycles per second.

#### 4.3.1.2 Amplitude

The amplitude of the wave is essentially the height of the wave. The amplitude of a wave can be described in a number of ways. Peak amplitude is the difference between the peak and average value of the wave. Peak-to-peak amplitude is the change between peak and trough of the wave; it is expressed as Vpp. Finally, RMS (root mean square) amplitude is a measure of the magnitude of a varying quantity. The RMS value of a sine wave is the square root of the average of the squares of the original values. For simplicity, the RMS value can be approximated by dividing the peak to peak amplitude by 2.8. RMS is a useful value as it can be used to calculate the average power of a sine wave and to calculate sound pressure level in decibels (dB SPL).

#### 4.3.1.3 Sound pressure

Sound pressure is the difference between the instantaneous pressure in the presence of a sound wave and the static pressure of the same medium with no

sound wave. In air, sound pressure can be measured with a microphone; in water it can be measured with a hydrophone. Sound pressure level (SPL) is a measure of the effective sound pressure compared to a reference value (on a logarithmic scale). SPL is measured in decibels (dB) above a standard reference level and is denoted as dB SPL.

4.3.1.4 Checking the sound waves

The sound waves created were checked using a digital storage oscilloscope (DSO). Repeating constant signals are displayed on the DSO as a static graph depicting the shape of the wave. The static graph generated allows the measurement of Vpp, frequency, and the time taken for a signal to rise to the maximum amplitude (signal onset or rise time). SPL was measured using a sound pressure level meter. Any distortions could easily be observed using a DSO.

## Methods

The techniques described in Chapter 2 were used to optimise the semi-automated startle assay in untreated animals. For full methods, see Sections 2.1, 2.9.1.3 to 2.9.5 and 2.2.9.7.

## Results

## 4.4 Calibrating the function generator for frequency and voltage

The predicted output of the Velleman 2.0 MHz PCGU1000 PC function generator was compared to the actual (measured) output to ensure that the instrument was correctly calibrated.

### 4.4.1 Frequency

The predicted and measured frequencies of the function generator matched exactly (Figure 4.1; linear regression: $r^2 = 1$, $DFn = 1$, $DFd = 13$), confirming that the function generator frequency output was as stated by the manufacturer.

### 4.4.2 Voltage

The predicted and measured voltage outputs of the function generator were equal (Figure 4.1; linear regression: $r^2 = 1$, $DFn = 1$, $DFd = 11$). This confirmed that the voltage output of the equipment was identical to the values stated by the manufacturer.

## 4.5 Determining the threshold for sine wave distortion over a range of frequencies

It was important to ensure that the pure tone sinusoids that were delivered to the control larvae had very little distortion. This was investigated by measuring the combined output of the function generator and amplifier in 400 mL of water using an oscilloscope coupled to a custom made hydrophone. Measurements taken at varying frequencies, voltages and volumes produced graphs to estimate the values at which the sine wave remained undistorted (Figures 4.2 a and b). Distortion was often measured at higher amplifier volumes and voltages, especially at the lower frequencies. The most commonly observed distortion was harmonic distortion with soft clipping, where the wave adopted an increasingly square or triangular characteristic (Figure 4.3). At the highest frequencies, voltage and volume were not sufficient to drive the signal towards distortion (Figure 4.2 b; (I), (K) and (L)). The data obtained from these initial measurements were subsequently used for each of the frequencies to test responses in the larvae (see Figure 4.4). Stimulus settings were later adjusted according to the optimal larval response and re-tested for non-distortion at the altered levels (see additional red and blue data points Figures 4.2 a and b).

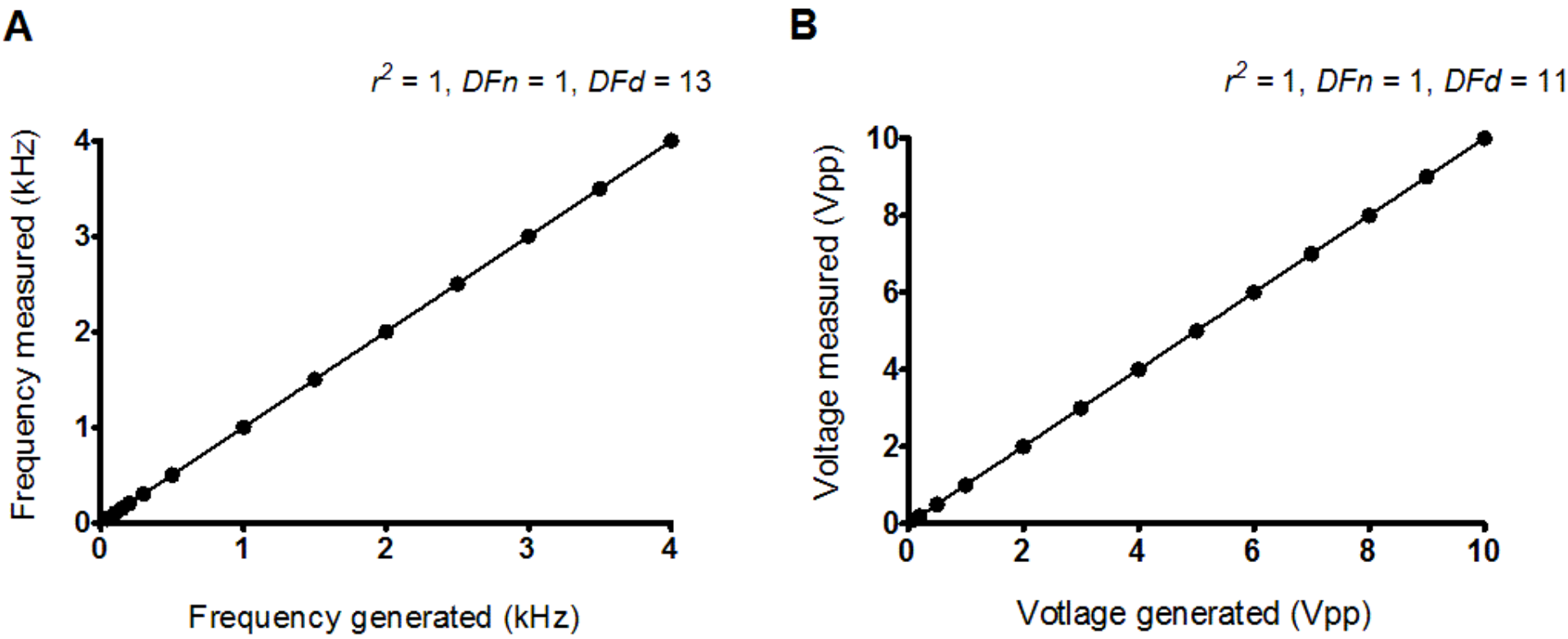



**Figure 4.1: Determining the accuracy of the function generator.** Measurements taken using an oscilloscope show that the measured output of the frequency generator equals the predicted output (directly proportional), for both frequency (A) and voltage (B).

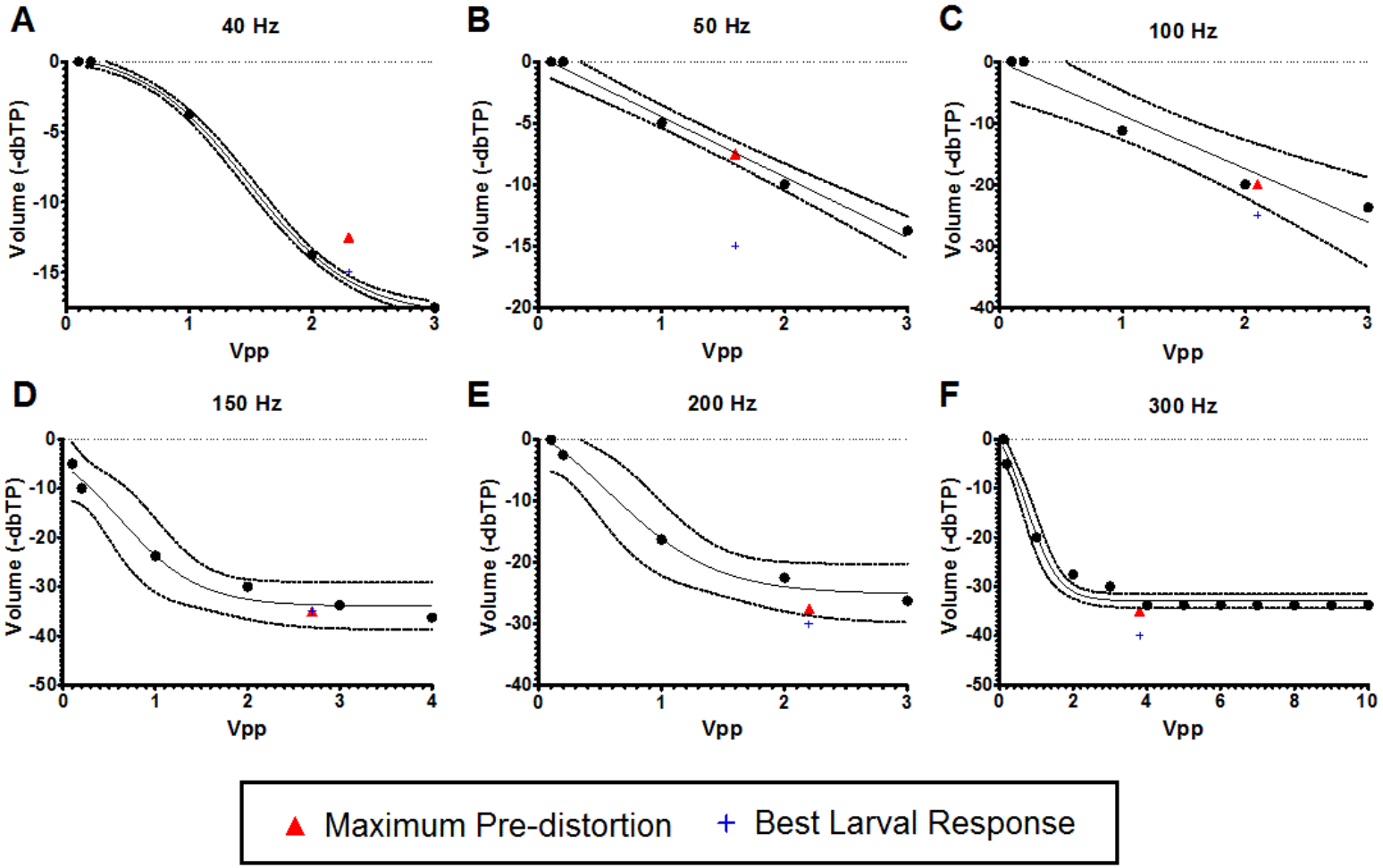



**Figure 4.2a: Testing for wave distortion thresholds over a 40-300 Hz frequency range.** Appropriate volume and voltage settings were chosen for each frequency (A-F). These were based on the approximated thresholds for distortion of the sinusoid. Settings were chosen that were predicted to form a pure tone. These settings were tested in the animals and adjustments made to the settings according to the best larval response. The best larval response (blue cross) was subsequently compared to the actual maximum volume settings (red triangle) for each frequency to ensure non-distortion. Data are representative of 2 repeat tests. Non-linear curve fitting has been applied. 95% confidence levels are indicated for each fit.

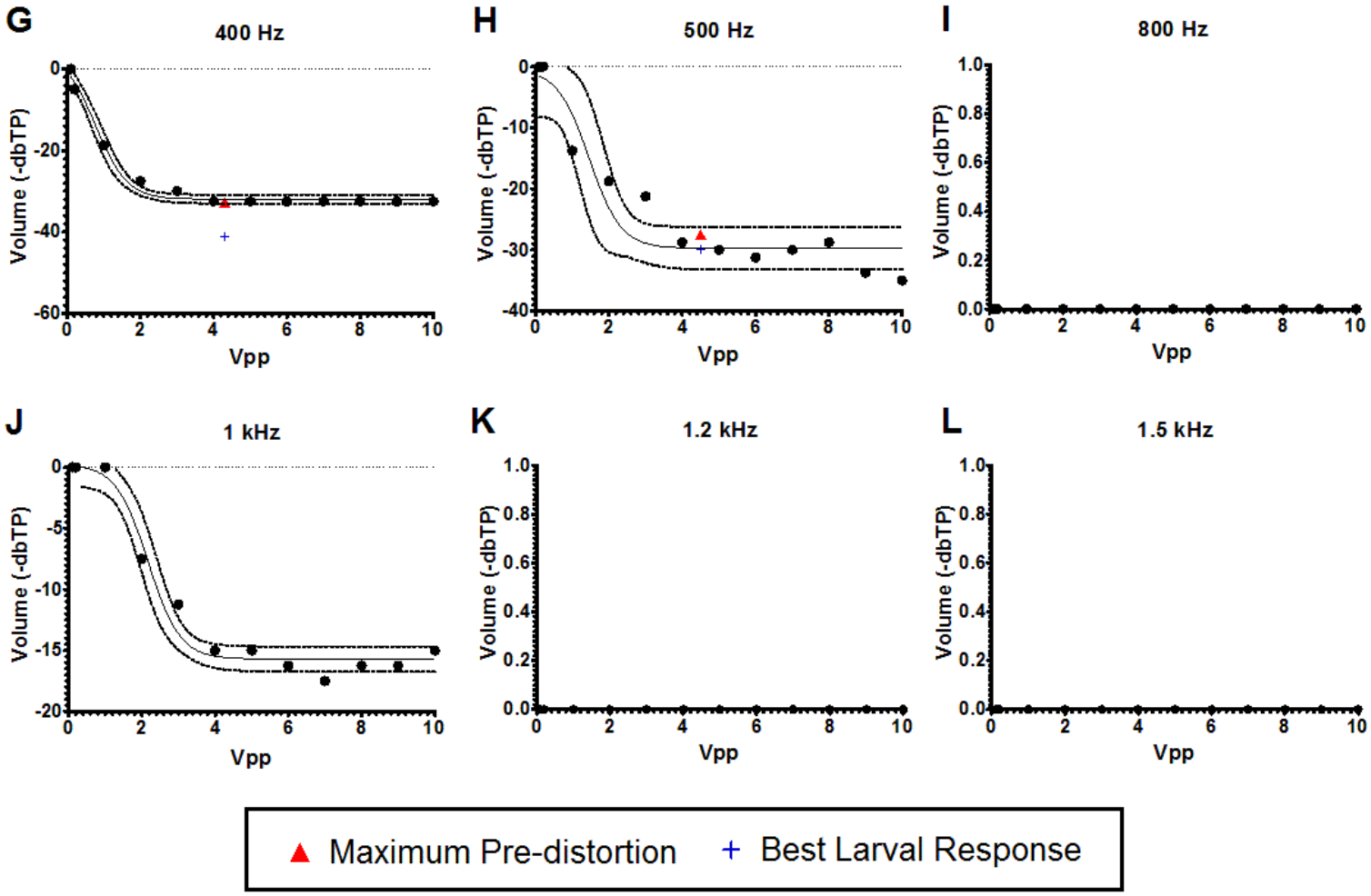



**Figure 4.2b: Testing for wave distortion thresholds over a 400-1500 Hz frequency range.** (G-L) Appropriate volume and voltage settings were chosen for each frequency and then tested in larvae, as described in figure 4.2a. Data are representative of 2 repeat tests. Non-linear curve fitting has been applied. 95% confidence levels are indicated for each fit.

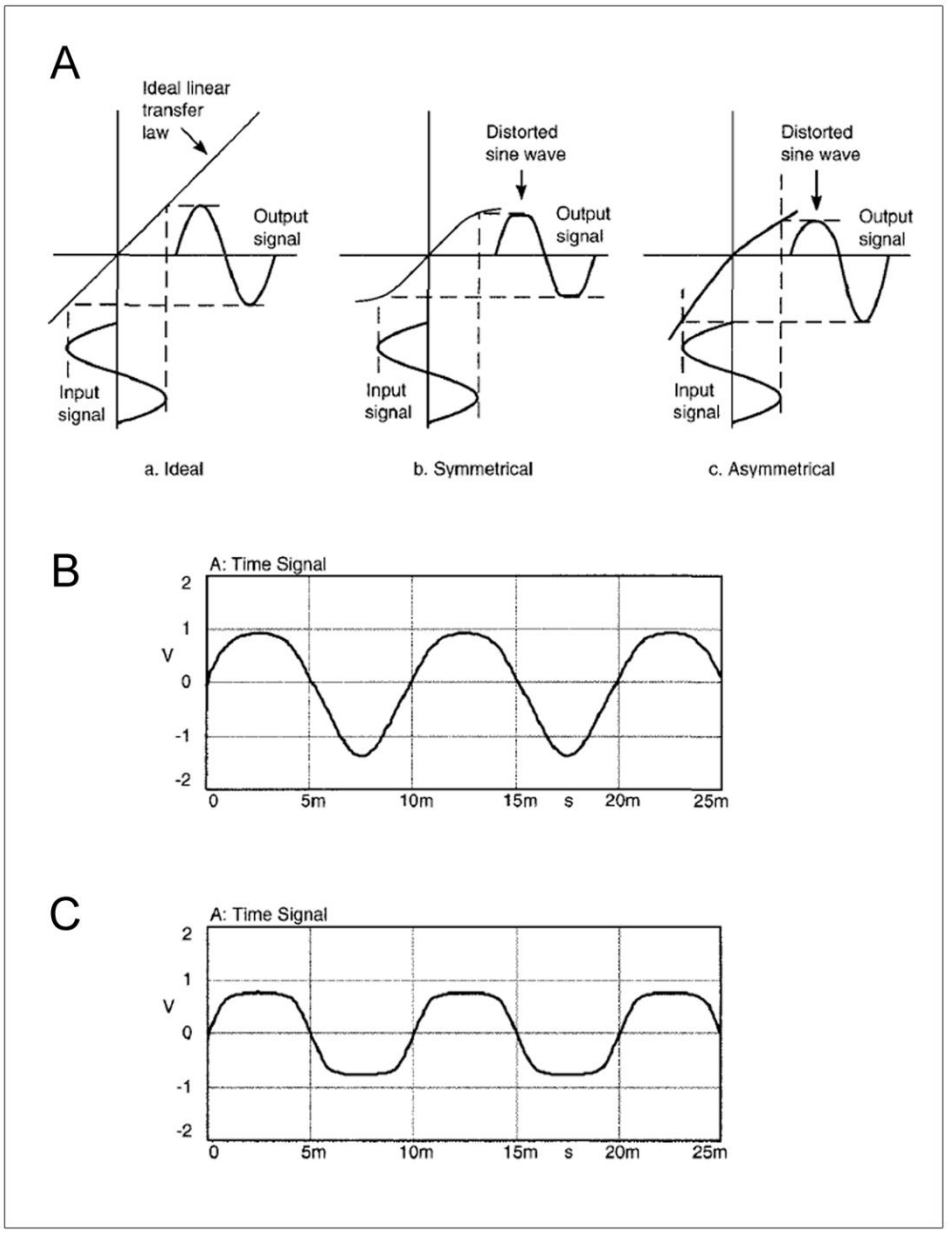



**Figure 4.3: Commonly observed wave distortions.** Image (A) depicts non-linear transfer characteristics. The image on the far left represents the ideal non-distorted wave, the central image shows a symmetrically distorted sine wave and the image on the far right shows an asymmetrically distorted sine wave. (B) An example oscilloscope trace showing the commonly observed positive peak limited sine wave. (C) An example oscilloscope trace showing the commonly observed positive and negative peak limited sine wave. Images courtesy of Steve Temme.

## 4.6 Initial testing of the startle response in larvae

### 4.6.1 Lighting conditions

Initially, larval startle responses were observed at a rate of 200 frames per second (fps). However, it was noted form the recorded video footage that the video displayed light/dark cycles on playback at this frame rate. The 'dark frames' made larval movement impossible to visualise by eye and to track using the Viewpoint software. The appearance of these light/dark cycles was the product of the frequency of the ambient AC lighting in the laboratory, which was found to be 120 Hz. In order to resolve this, a capture rate of 60 fps was adopted, which was half the frequency of the lighting. At this frame rate, it was possible to visualise larval startle responses without on/off flicker.

### 4.6.2 Fluid volume

The effect of using smaller fluid volumes in each well of the microplate was investigated with the aim of minimising the amount of compound required for each test. It was found that when a fluid volume of 1 mL or less was used, it was not possible to accurately visualise the fish that were performing thigmotaxis (swimming at the edge of the wells; Schnorr et al., 2012) as the wells were too deep and the camera too far from the microplate. Consequently 2 mL of fluid per well was selected for the final assay. At this volume, it was possible to visualise the larvae across the entire well. Qualitatively, larval startle responses appeared unaffected by altering the fluid volume from 1 to 2 mL.

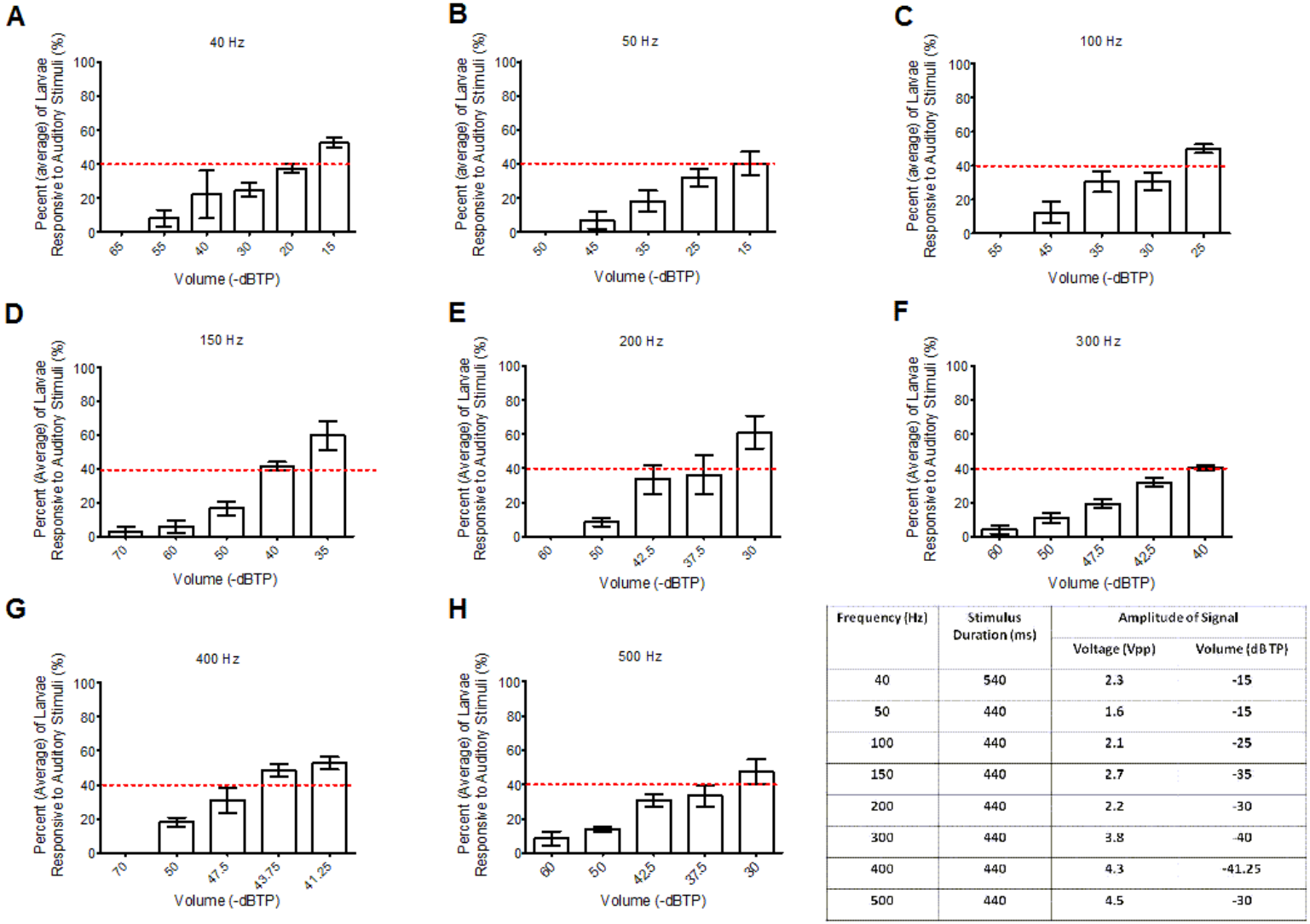
### 4.6.3 Stimulus duration

The duration of the sinusoidal stimulus was restricted to the minimum duration that the software could offer (denoted as '1 second') for all but one of the stimuli. The larvae were able to respond with a startle to the shortest stimulus. Qualitatively, there was no improvement in the response shown to longer stimuli, as the animals were responding only to the onset of the signal. At the lowest frequency (40 Hz), a '1 second' stimulus was inaudible and ineffective at producing a startle response. It was therefore necessary to increase the stimulus duration to '1.1 seconds' in order to elicit the response although the actual length of the stimulus appeared to be less than 1-1.1 seconds. Direct measurements of the '1 second' and '1.1 second' tone bursts with the

oscilloscope revealed that the actual stimulus durations were 440 ms and 540 ms respectively.

## 4.7 Determination of the threshold responses of larvae to a range of pure tone stimuli

For all frequencies, the threshold for indicating the successful induction of a startle (minimum startle responsiveness) was 40% (>40% of fish responding to stimulus). A summary of the manual scoring data are shown in Figure 4.4. The optimal settings for larval AER are plotted in Figures 4.2 a and b (blue cross). The highest frequency that elicited an AER that could be tested was 500 Hz. No reproducible larval response could be elicited above this frequency. The greater power (voltage and volume) required to drive stimulation at higher frequencies evoked a double startle as the speaker audibly clicked on and off. In addition, fewer than 40% of larvae per plate responded to even the loudest stimulus before distortion of the waveform occurred at frequencies above 800 Hz.




| Frequency (Hz) | Stimulus Duration (ms) | Amplitude of Signal | |
|---|---|---|---|
| | | Voltage (Vpp) | Volume (dBTP) |
| 40 | 540 | 2.3 | -15 |
| 50 | 440 | 1.6 | -15 |
| 100 | 440 | 2.1 | -25 |
| 150 | 440 | 2.7 | -35 |
| 200 | 440 | 2.2 | -30 |
| 300 | 440 | 3.8 | -40 |
| 400 | 440 | 4.3 | -41.25 |
| 500 | 440 | 4.5 | -30 |

**Figure 4.4: Initial testing of frequency settings in control animals.** (A-H) Estimated settings for voltage (Figure 4.2 (a) and (b)) were tested at each frequency in short tone bursts. Volume settings were altered to determine the best larval response achievable. Red dotted line represents the 40% threshold. Frequencies of 800 Hz and above could not be used, as explained in the text. The data displayed represent the average of 3 repeated stimuli per volume setting, with error bars showing SEM. Settings that elicited the best larval response were re-tested to ensure non-distortion of the sinusoid (Figure 4.2 (a) and (b)). The optimised voltage and volume settings for each frequency are shown in the table (inset).

## 4.8 Re-testing of the optimised stimuli

### 4.8.1 Re-testing for distortion in a 400 mL fluid volume

Following assessment and optimisation in the larvae, the chosen voltage and volume settings for each frequency were retested using the same method as in Section 2.9.2.1. This additional check was performed because some larvae required a louder volume than predicted by the line/curve fitting in order to elicit an adequate response. This additional measurement is marked by the red triangle (Figures 4.2 a and b) and is an accurate maximum value before wave distortion occurred. As shown in these figures, none of the values used for the final assay (denoted 'best larval response') exceeded the maximum value before distortion.

### 4.8.2 Testing for distortion in the 24 well microplate

There was little to no distortion of the wave when the final settings were examined for volume, voltage and frequency in well 4C of the plate using a microphone connected to the oscilloscope.

### 4.8.3 Testing the spread of signal across the 24 well microplate

In order to ensure that there were no edge effects, it was desirable to assess the spread of the signal across the microplate. Initially, a small omni-directional microphone was used in each well of the plate to detect any variation in sound pressure level or wave distortion, when coupled to an oscilloscope. However, due to the physical limitations of the equipment, it was not possible to take measurements from each individual well. As a simple and safe alternative, sodium chloride powder was employed to visualise the pattern of sound waves in the plate in the absence of water (see Supplementary Material Video "salt test". The spread of the signal appeared uniform as the salt moved in each of the 24 wells (see Figure 4.5 for a representative image).

### 4.8.4 Calibration of sound pressure level

Figure 4.6 shows the data from SPL calibrations at each frequency. Overall, there was a correlation of measurement between both media (water and air) at individual fixed frequencies (40-500 Hz). SPL meter readings were positively correlated with the readings taken in both air and water (as the SPL meter reading increased, so did the SPL calculated from the microphone recordings). As predicted, there was a difference in microphone SPL measurements in air versus water for each frequency tested; this varied from 14-24 dB SPL. Interestingly, there was an increase in the SPL recorded with the microphone as the frequency setting decreased, when compared with the SPL meter. This was unexpected and is explained by the resonance of the apparatus, which could not be detected by the SPL meter in air, but was detectable in water using the microphone. This resonance did not appear to distort the wave created by the speaker.

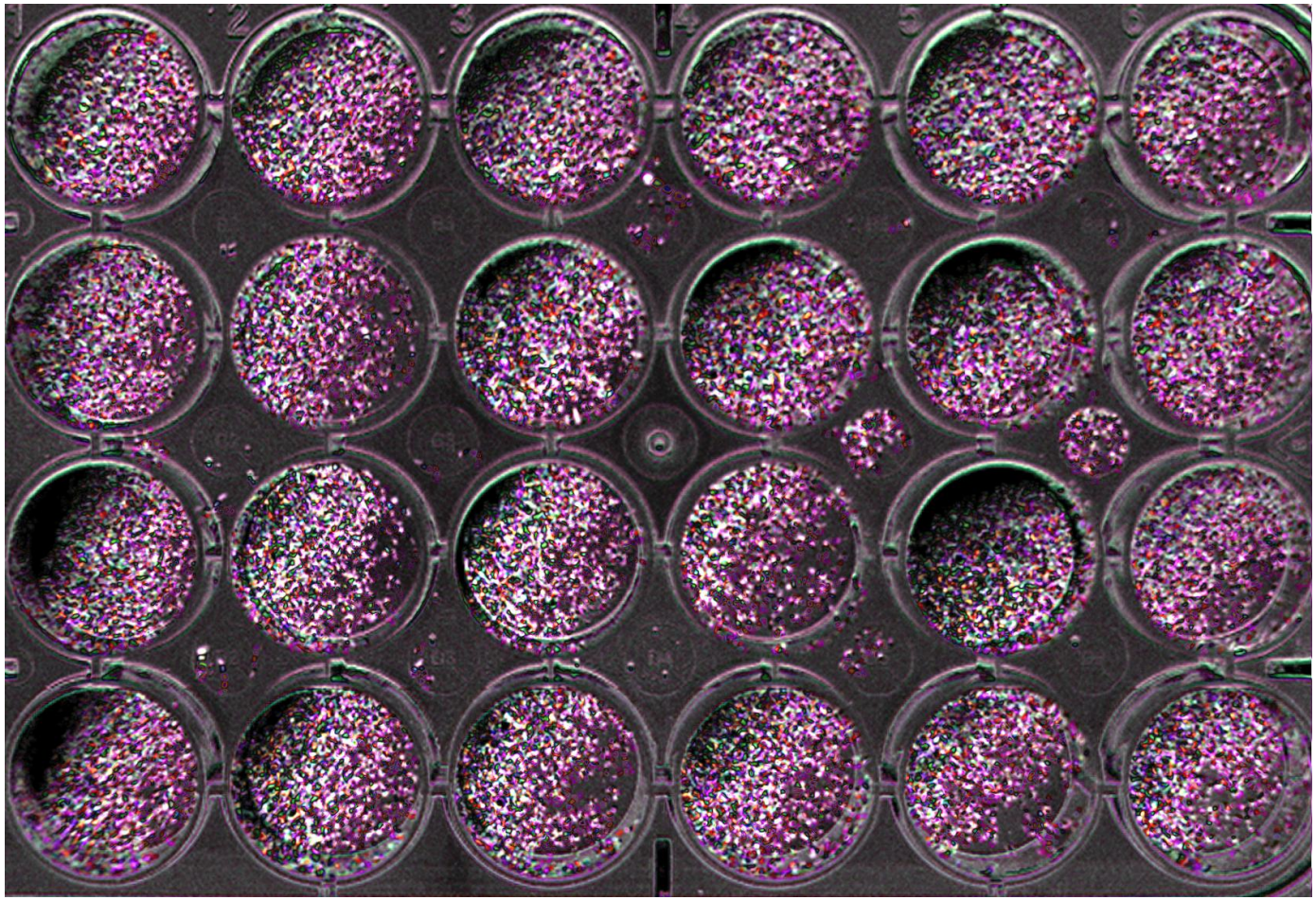


**Figure 4.5: The spread of signal across a 24 well microplate.** This time-to-colour merge picture was generated from frames 877-882 of the “Salt test” video (Supplementary Material). Grey scale shows lack of movement, colour indicates movement. Overall, the spread of movement looks even across the plate. Note: dark patches within the wells are due to light reflection and high density salt patches; movement colour outside of the wells is due to salt escaping from the wells.

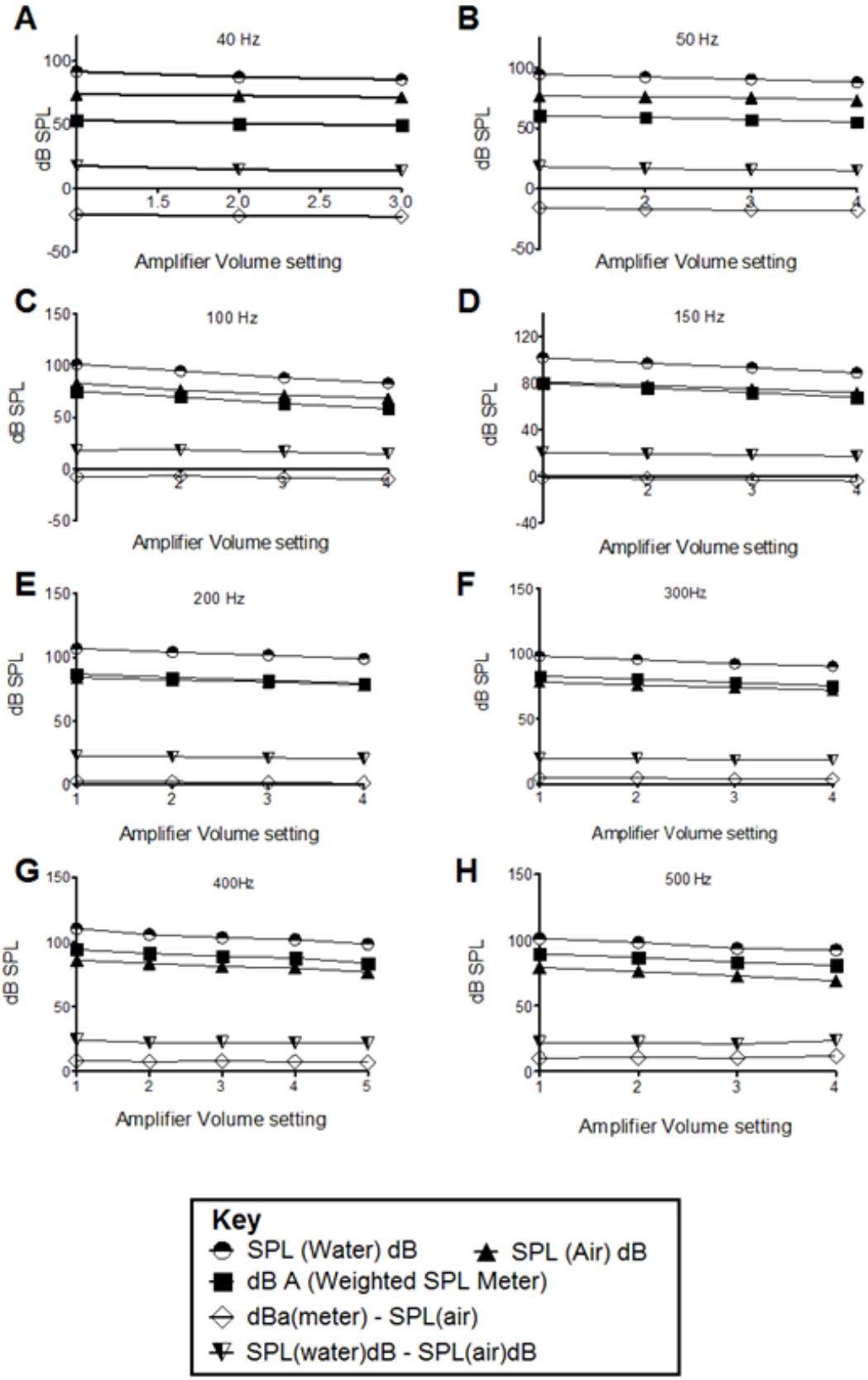



**Figure 4.6: The relationship between sound pressure level measurements and microphone SPL in air versus water.**

## 4.9 Setting the threshold speed of the stereotypical startle response

After initially testing for startle threshold on one video at a number of speeds, further testing was undertaken on three thresholds that were 5.5 mm $s^{-1}$ apart. This involved replaying and scoring videos by eye more than 1440 times. Data for the manual comparison of the 3 alternative thresholds for high-speed AER are shown in Figures 4.7 to 4.9. The thresholds evaluated were 15.6 mm $s^{-1}$, 21.1 mm $s^{-1}$ and 26.6 mm $s^{-1}$. Two average values are reported that indicate the level of concurrence between score by eye and Viewpoint traces; one overall value that takes into account all animals (including non-responders; 'overall percentage match' (OPM)) and one value that only takes into account traces for movement and startle (excluding non-responders; 'movement and startle only' (MSO)). Assessments using Viewpoint colour traces over 20 stimuli showed that changing the high-speed threshold had a significant effect on OPM (Figure 4.7 B; Repeated Measures ANOVA followed by Tukey's multiple comparison test: $F(2, 19) = 32.68$, $P < 0.0001$) and MSO (Figure 4.7 D; Repeated Measures ANOVA followed by Tukey's multiple comparison test: $F(2, 19) = 34.19$, $P < 0.0001$). The most appropriate thresholds for detection of the high-speed escape response appeared to be 21.1 mm $s^{-1}$ (OPM = 86%; MSO = 85%) and 26.6 mm $s^{-1}$ (OPM and MSO = 89%). Post-testing showed no significant difference in percentage match at these two levels (Tukey's multiple comparison test, $P > 0.05$), whereas a setting of 15.6 mm $s^{-1}$ gave a significantly reduced percentage match with the score by eye compared with the two higher thresholds (Tukey's multiple comparison test, $P < 0.05$). Tracking of the motion was much less stringent at 15.6 mm $s^{-1}$; often movements (M) were displayed as mild startles (MS) on playback through the Viewpoint system. Conversely, limiting the threshold of the response to 26.6 mm $s^{-1}$ was excessively stringent; fish that had been scored as performing a mild startle (MS) by eye were displayed as (M) only with tracking (see examples Figure 4.8; G and H). A threshold of 21.1 mm $s^{-1}$ had a higher percentage match than 15.6 mm $s^{-1}$ in 19 out of 20 of the videos (OPM = 86% versus 72%; MSO = 85% versus 68%). 21.1 mm $s^{-1}$ was chosen as the final threshold for the assay. At this level, most mild startles could still be detected and many of the non-startle movements were not being mistaken for an escape response (see Figure 4.9).

## 4.10 Comparing results of the automated tracking software to scoring by eye

A direct comparison between scoring responses by eye and the data generated by the viewpoint software set to a threshold of 21.1 mm $s^{-1}$ showed an excellent concurrence over the 20 sample recordings. The overall percentage match (OPM) was 85.6%. The percentage match for tracking of movement and startle only (MSO) was 83.3%. Reasons for the incompatible scores will be discussed in more detail in Section 4.19.2. There was no significant difference between the manual visual traces produced using Viewpoint and the automated data the software created for the 21.1 mm $s^{-1}$ threshold (Figure 4.10; OPM t-test: $t$ = 0.4197, *d.f.* 19, $P$ = 0.6794; MSO t-test: $t$ = 1.050, *d.f.* 19, $P$ = 0.3069). This suggests that the inclusion of additional tracking before and after the stimulus (caused by the 1 second time binning) should not influence the outcome of the analysis.

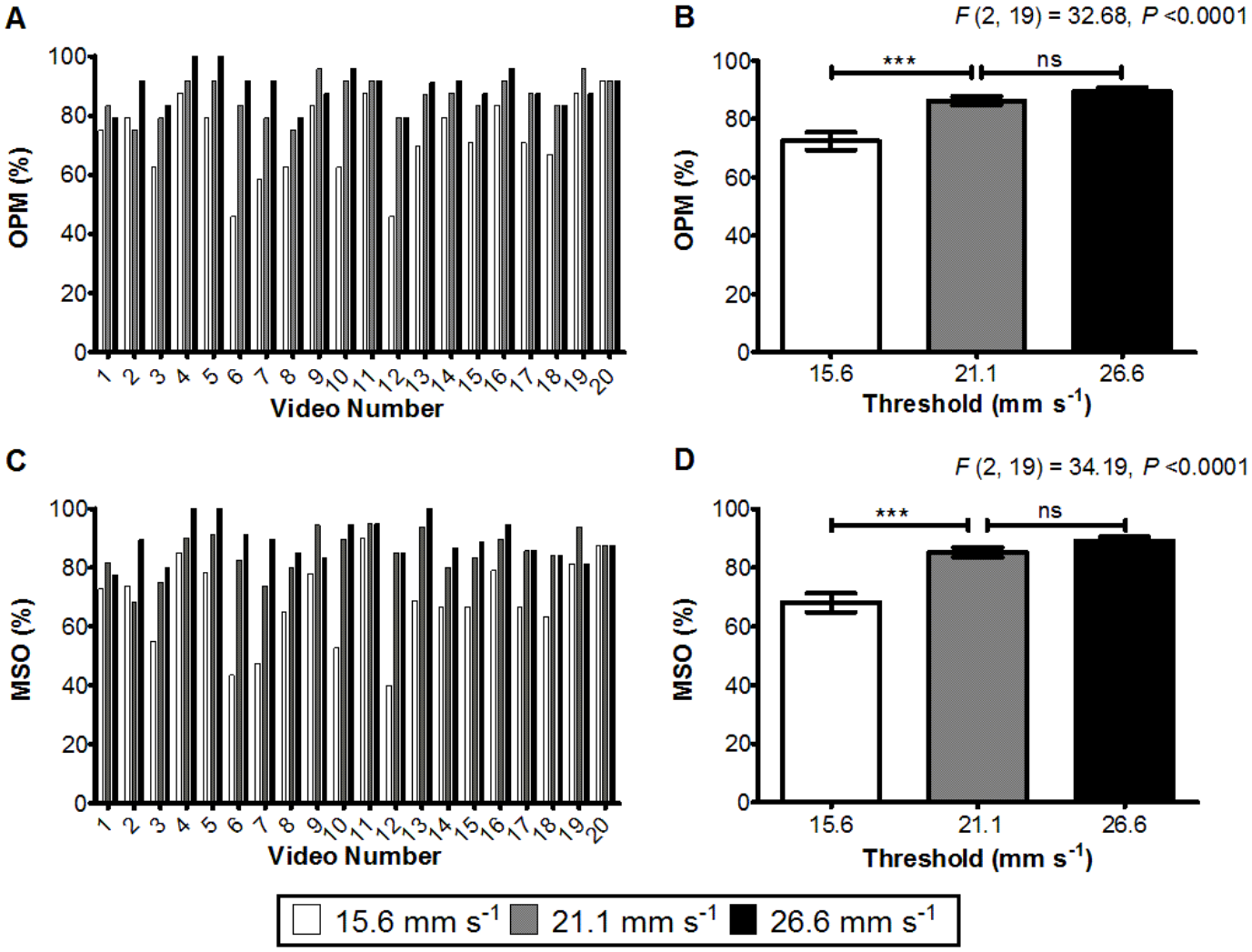



**Figure 4.7: Selecting the optimal threshold to identify the stereotypical startle response.** The results from three speed thresholds were compared over 20 video recordings to identify the best setting for accurate startle detection. Videos were scored by eye and compared to viewpoint colour traces (see Fig. 4.8). Values displayed are for overall percentage match (A and B) and matching for movement and startle only (C and D). (B) and (D) Data displayed are an average taken over 20 videos, with error bars representing SEM. Adjusting the high-speed threshold has a significant effect on OPM and MSO in control animals. Results of all videos individually are shown in (A) and (C).

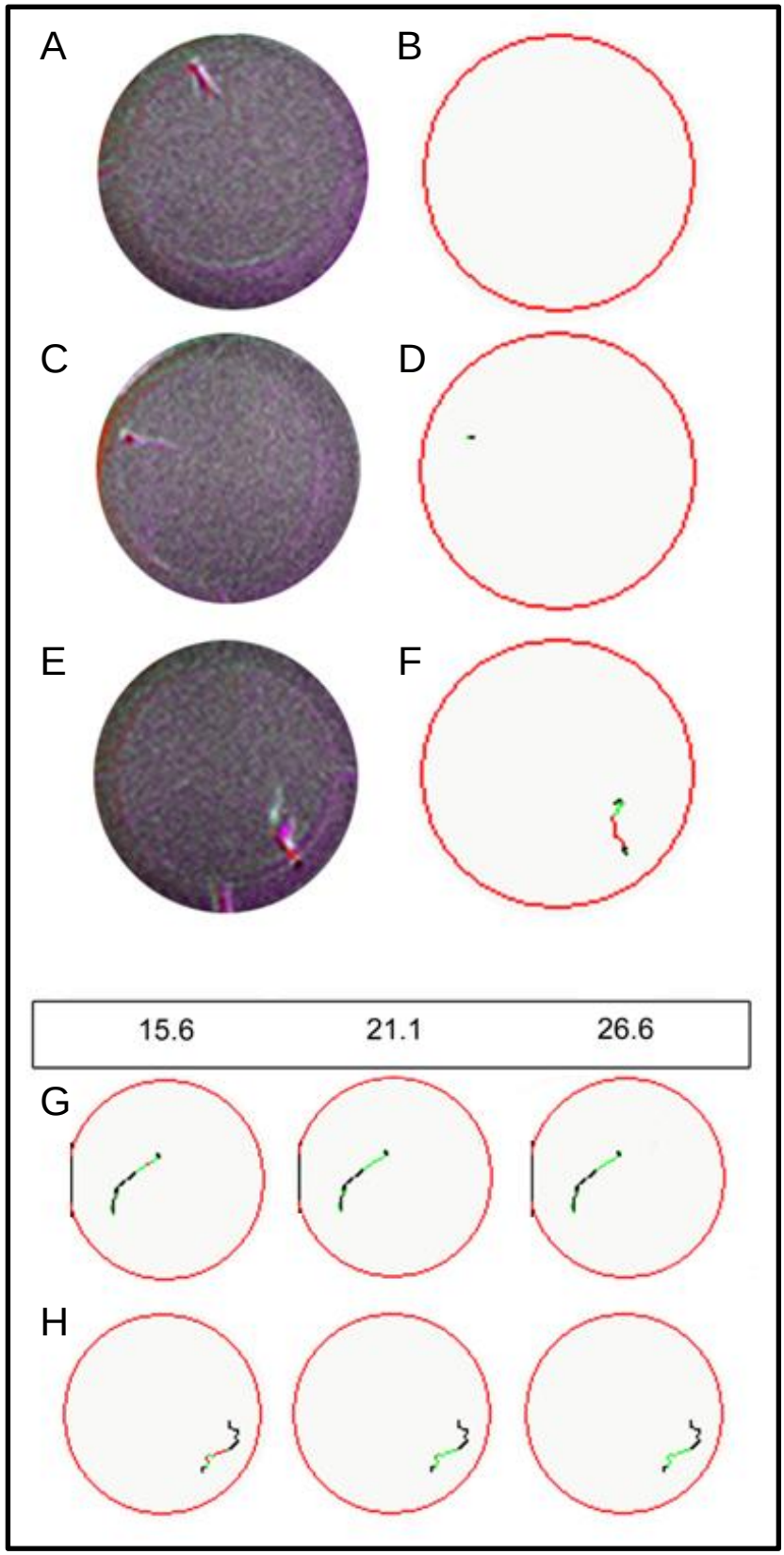



**Figure 4.8: Example Viewpoint tracking. (A-F) Occasionally animals are not detected, despite movement.** (A, C and E) Time-to-colour merge pictures generated from frames 379-384 of video 14 “habituation testing extremes rep 3 500 Hz AVI 8” (Supplementary Material). Grey scale shows lack of movement, colour indicates movement. (B, D and F) Viewpoint automated tracking from video 14 (A), (B) Images generated from well D3. The larva moves but is not detected.(C), (D) Images generated from well C6. The larva does not move but is detected at its centre of gravity. E), (F) The larva moves and is detected by tracking. **(G-H) Differences between traces over three test thresholds.** (G) Example traces for an “M” score by eye.(H) Example traces for an “MS” score by eye.

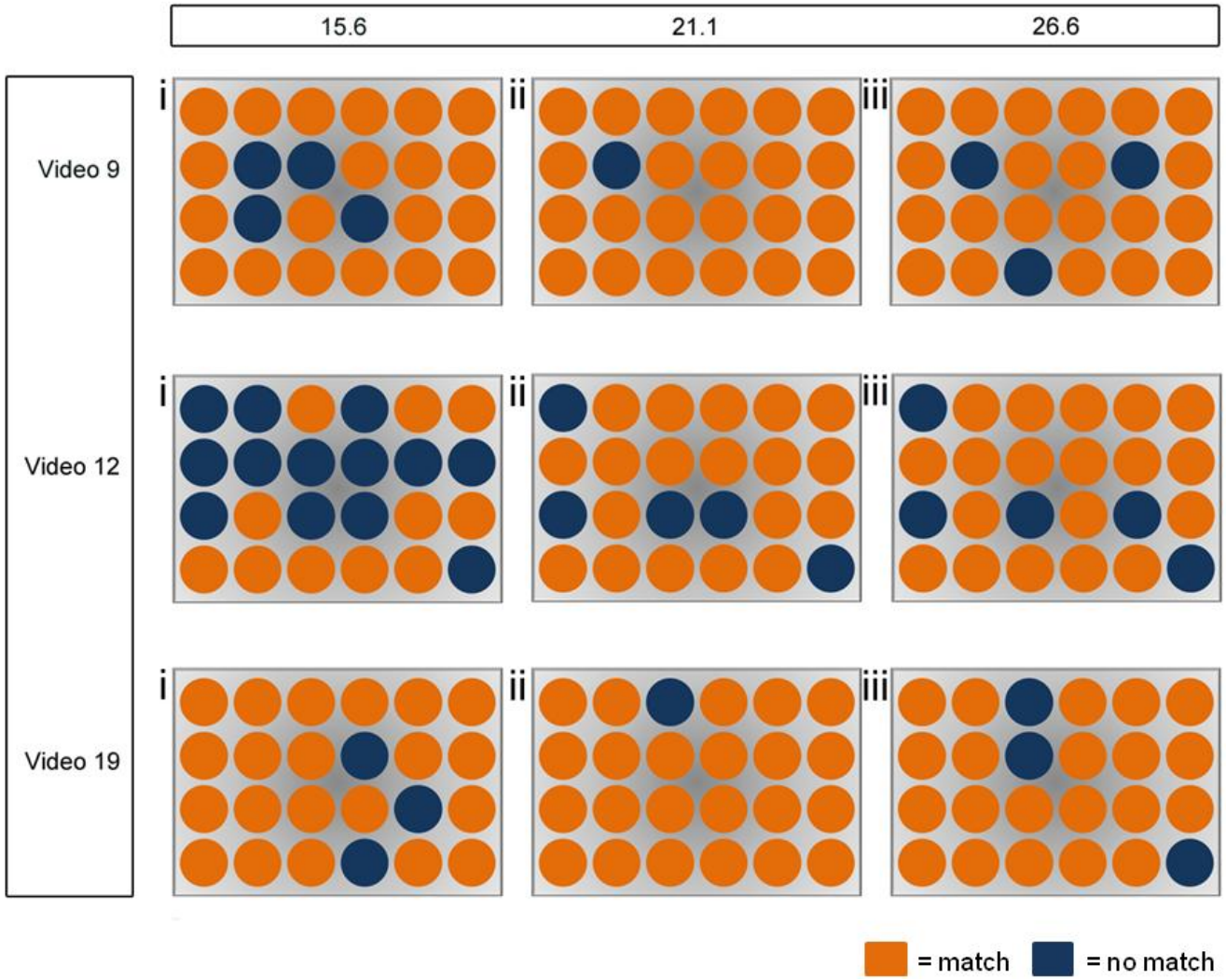



**Figure 4.9: Selecting the optimal threshold to identify the stereotypical startle response.** Images display the concurrence between Viewpoint traces and score by eye at each of the thresholds. In videos 9, 12 and 19, 21.1 mm $s^{-1}$ is the most accurate indicator of the startle response. 15.6 mm $s^{-1}$ is the least accurate threshold to identify startle responses, often lacking stringency (video 12(i)).

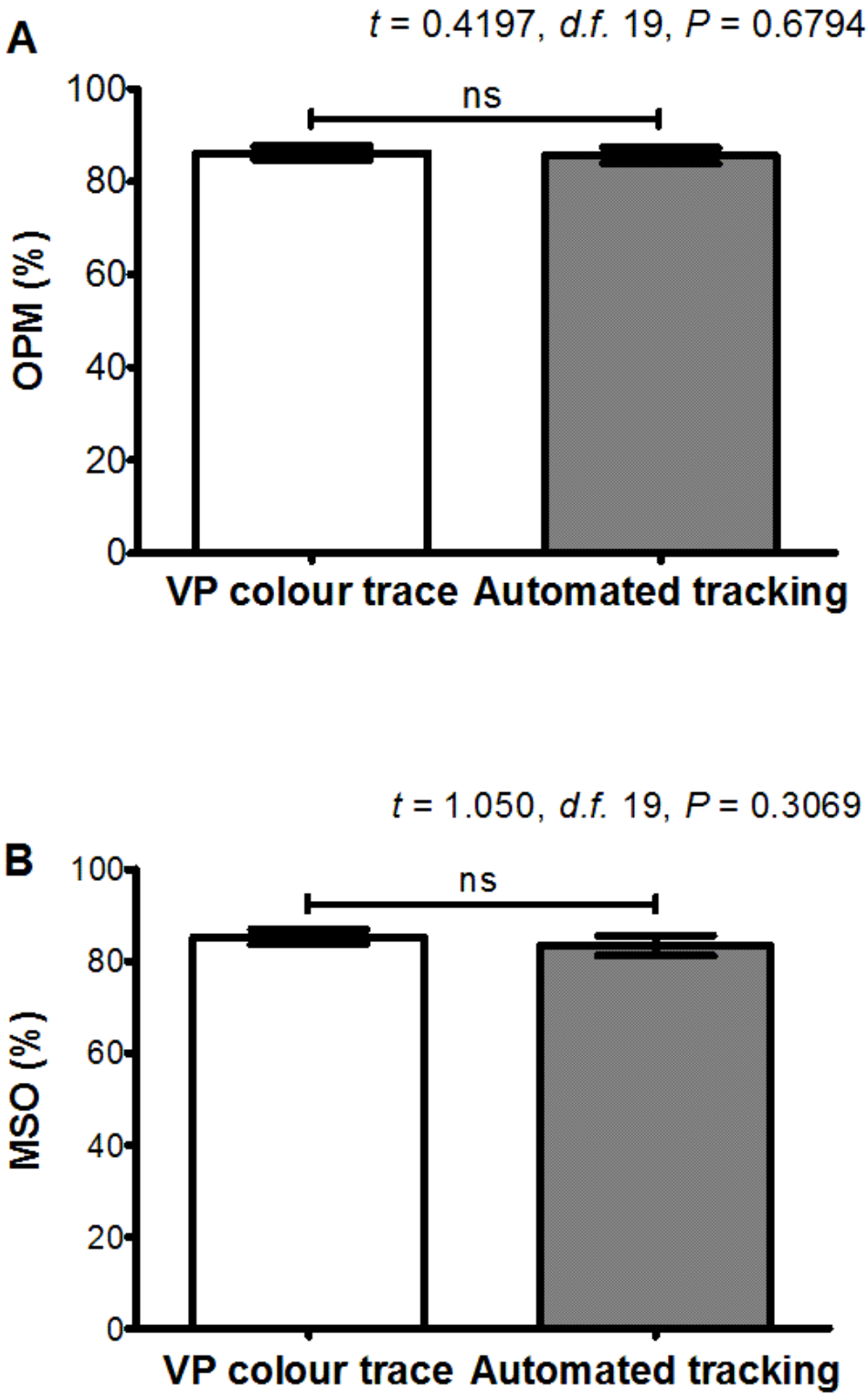



**Figure 4.10: Comparing results of Viewpoint colour traces and automated tracking using the VideoTrack software.** A comparison of viewpoint traces and automated tracking software at the chosen threshold of 21.1 mm $s^{-1}$. There was no significant difference between traces and automated tracking for either OPM (A) or MSO (B). The data points shown are the average over 20 stimuli. Error bars represent the SEM.

## 4.11 Using Excel to detect the timing of the startle response automatically

A macro was created in Excel to remove inactive larvae and outlying data and to isolate the point in the 10 second recording in which the stimulus was delivered/the startle response occurred. Data were only excluded if they were ± 2 standard deviations from the mean. An example of the graph created can be seen in Figure 4.11 A. The graph not only highlights the timing of the startle response, but also provides an 8-9 second baseline recording of any movement within the plate that was above 21.1 mm $s^{-1}$. This macro was essential in the further processing of the data and its accuracy was of great importance. Over the 20 sample videos, I found a 100% match between the automated detection of startle and the actual onset of the response (Figure 4.11 B).

## 4.12 Factors influencing control compliance

### 4.12.1 Plate type

As summarised in Figure 4.12, plate format (24- vs. 48-well) had a significant effect on the startle response, with larvae in the 24-well plate displaying a stronger startle response overall (two-way ANOVA; $F$ (1, 32) = 8.72, $P$ = 0.0058). The frequency of the stimulus had no effect on response strength (two-way ANOVA; $F$ (7, 32) = 0.72, $P$ = 0.6529). It is noteworthy that plate format did not have the same effect at all frequency values (two-way ANOVA; $F$ (7, 32) = 0.66, $P$ = 0.7028). Taken together, these data supported the use of 24-well plates in the assay to ensure the most reproducible reactions.

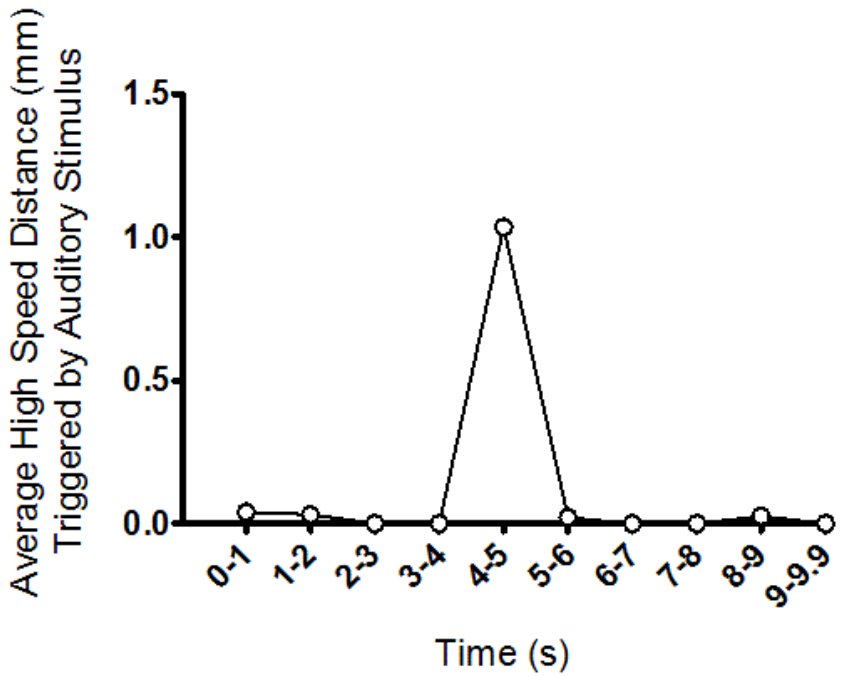
### 4.12.2 Wave type

It was also investigated whether an alternative stimulus wave shape (square) could elicit an improved larval response compared with a sinusoid. Both sinusoidal and square waveforms produced equally strong AERs (Figure 4.13 A; Paired t-test: t = 0.6267, 22 d.f., P = 0.5373), with no difference in the proportion of responding larvae (Figure 4.13 B; Paired t-test: t = 0.6146, 23 d.f., P = 0.5449). I therefore chose to use the more conventional sine wave for the final startle assay design.

### 4.12.3 Raising density

Raising larvae at a density of 50 per Petri dish produced a significantly stronger startle movement than raising animals at 128 per dish (Figure 4.14 A; 2.09 ± 0.13 (50/dish) compared with 1.62 ± 0.16 mm $s^{-1}$ (128/dish); *Mann Whitney U Statistic* = 1743, 1 *d.f.*, $P$ = 0.0033). Larvae raised at the lower density displayed increased responsiveness to the stimulus when compared with those reared at the higher density (Unpaired *t*-test on transformed data; Figure 4.14 B; 0.85 ± 0.044 compared with 0.64 ± 0.039; $t$ = 3.707, 142 *d.f.*, $P$ = 0.0003). The developmental progression and health of the larvae, however, did not appear to be changed between the different rearing conditions. Qualitative assessment revealed that larvae raised at both densities inflated their swimbladders, and developed normal-sized jaw, ear and eye structures and a functional digestive tract (Figure 4.14 C).

A




B

| Video Number | Time of startle (s) | Frame | Automated time bin allocation (s) | Match? |
|---|---|---|---|---|
| 1 | 3.9 | 235 | 3-4 | yes |
| 2 | 1.0 | 60 | 1-2 | yes |
| 3 | 3.0 | 180 | 3-4 | yes |
| 4 | 4.0 | 242 | 4-5 | yes |
| 5 | 5.0 | 298 | 5-6 | yes |
| 6 | 6.3 | 380 | 6-7 | yes |
| 7 | 7.7 | 465 | 7-8 | yes |
| 8 | 4.9 | 292 | 4-5 | yes |
| 9 | 5.0 | 302 | 5-6 | yes |
| 10 | 4.6 | 278 | 4-5 | yes |
| 11 | 3.7 | 222 | 3-4 | yes |
| 12 | 5.9 | 356 | 5-6 | yes |
| 13 | 5.2 | 314 | 5-6 | yes |
| 14 | 6.3 | 377 | 6-7 | yes |
| 15 | 4.8 | 289 | 4-5 | yes |
| 16 | 6.7 | 402 | 6-7 | yes |
| 17 | 3.6 | 219 | 3-4 | yes |
| 18 | 3.8 | 227 | 3-4 | yes |
| 19 | 4.2 | 125 | 4-5 | yes |
| 20 | 3.5 | 210 | 3-4 | yes |
| | | | Percent match (%) | 100 |

**Figure 4.11: Identifying the stimulus time using macro design.** (A) An example figure showing the result of macro processing from a single stimulus over a 10 second recording (taken from neomycin trial a plate 1 200 Hz). The stimulus was delivered and an AER elicited at 4-5 seconds. (B) A comparison of the results generated by the macro versus the original video recordings. There is a 100% agreement between predicted stimulus time and observable startle.

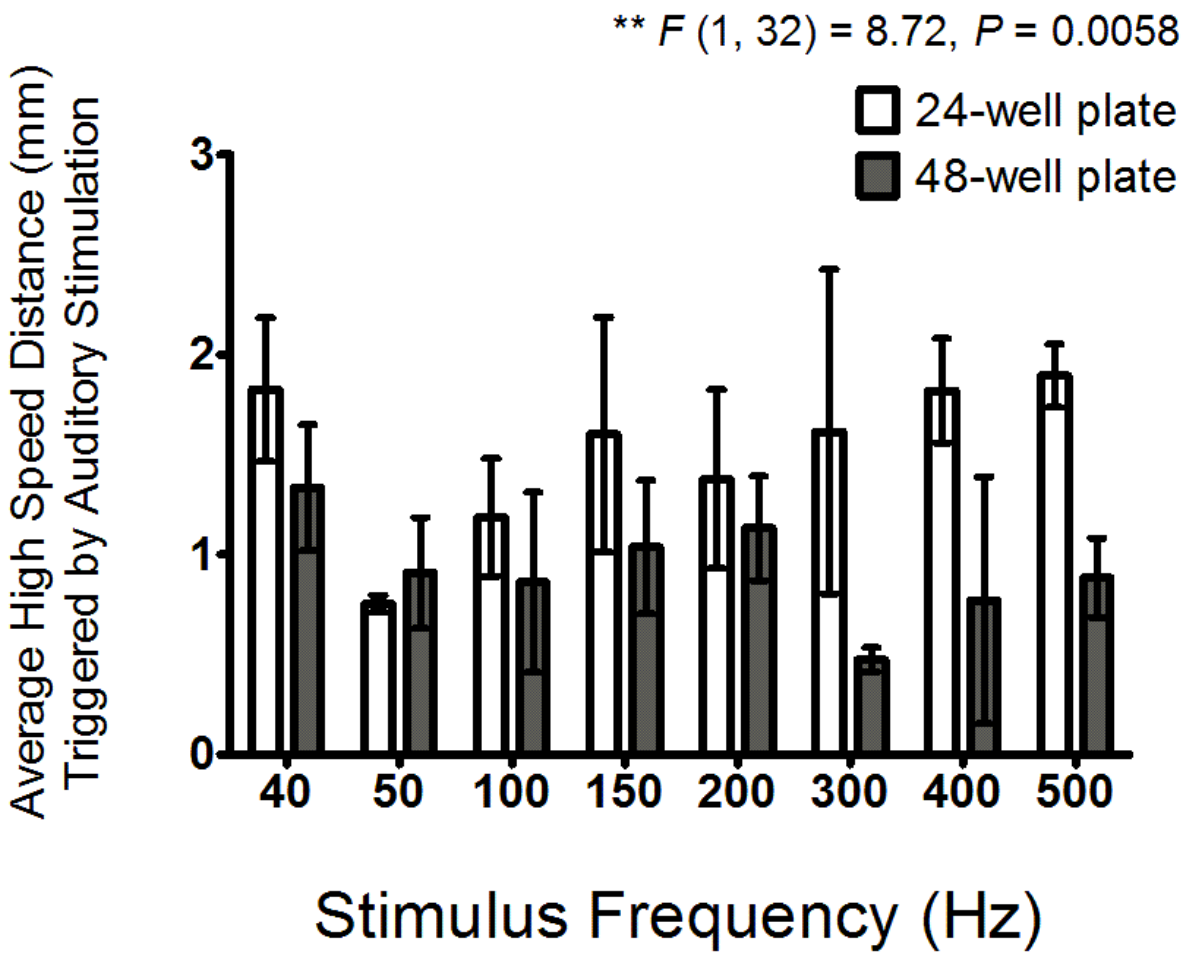



**Figure 4.12: Plate type contributes to the control compliance of larval AERs.** Overall, untreated larvae displayed stronger AERs when arrayed into microplates containing 24 wells compared with 48 wells (two-way ANOVA). The average value from three trials is shown for each plate format and frequency. Error bars represent SEM.

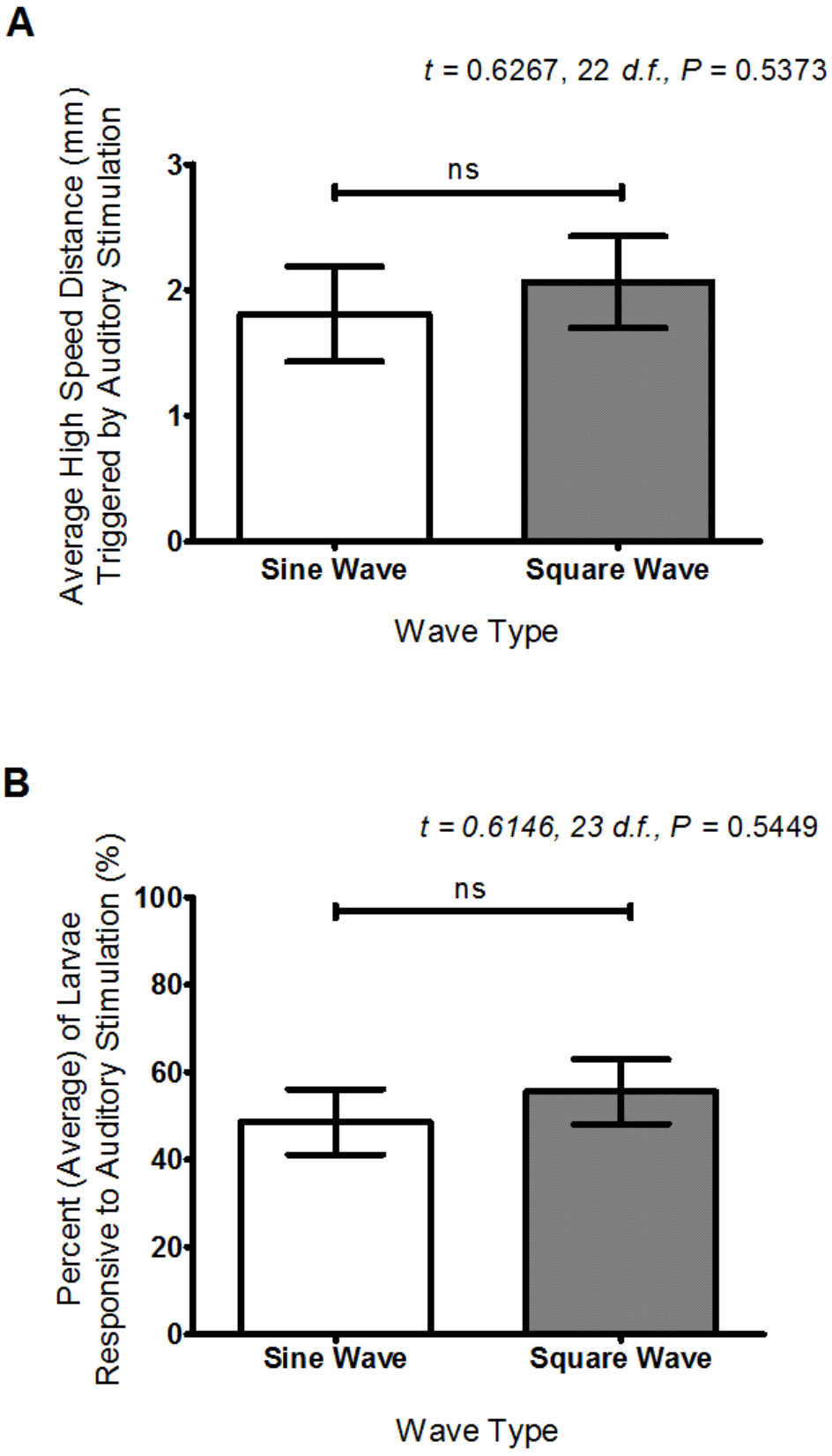



**Figure 4.13: Wave type does not affect larval AERs.** (B, C) Paired t-tests indicated that both sine and square waveforms elicited equally strong startle responses (based on the strength of response and the percentage of animals responding). Data shown are the average values from a total of 3 repeated trials. Error bars show SEM.

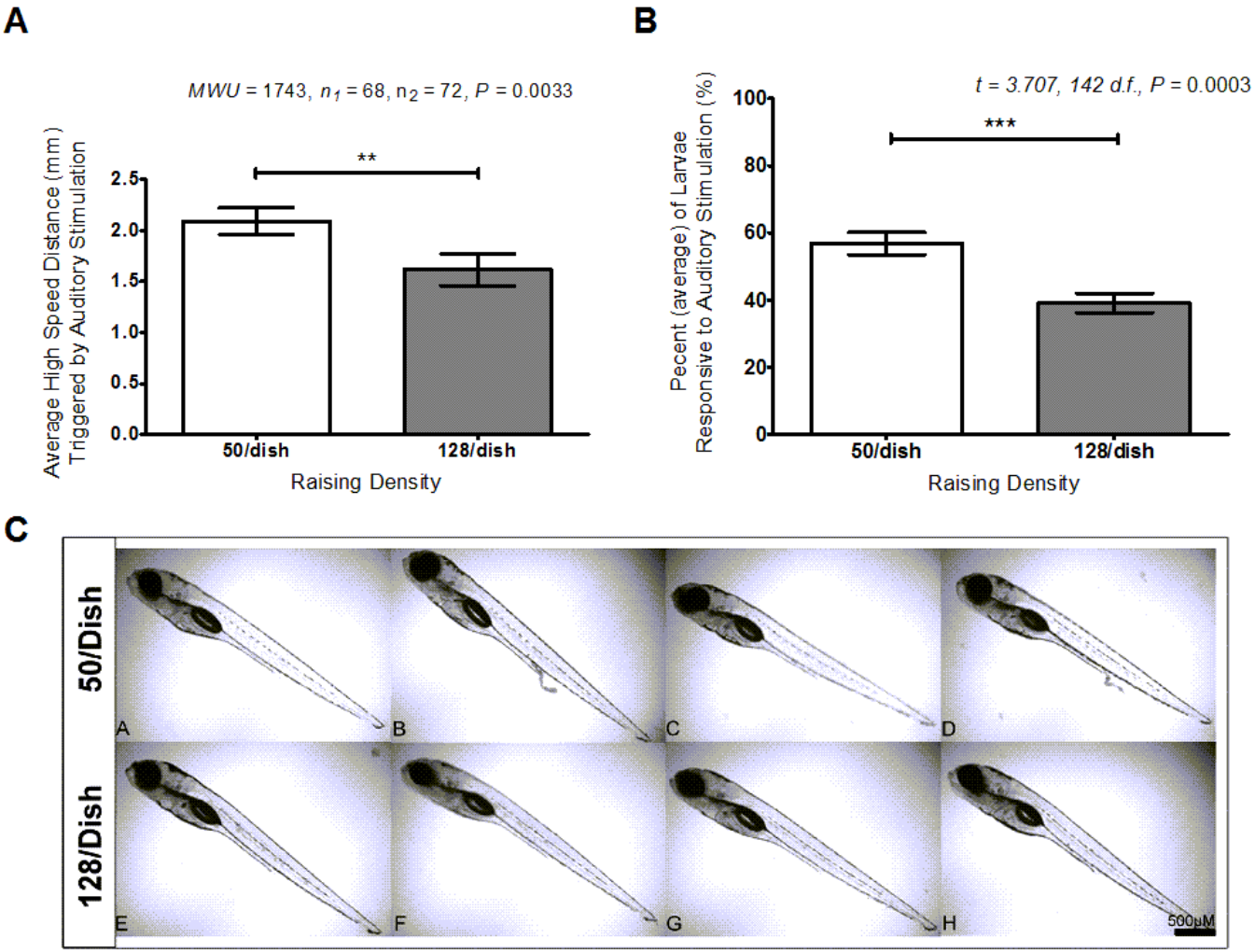



**Figure 4.14: Density of raising affects larval AERs.** (A, B) A density of 50 larvae per Petri dish gave both the strongest startle response (Mann Whitney test) and the highest percentage of untreated larvae startling (unpaired t-test), when compared with a higher density of 128 larvae per Petri dish. Data shown are the mean average value of three trials, with error bars showing SEM. (C) Development was unaffected by altering the density at which larvae are raised. (a-d) Representative images of larvae reared at a density of 50 per Petri dish (taken from a sample of 150 animals). (e-h) Representative images of larvae reared at a density of 128 per Petri dish (taken from a sample of 128 animals). Note normal development of eye, body pigmentation, body length, and swimbladder inflation for all animals. Scale bar = 500 µm.

### 4.12.4 ISI

To determine if there was any habituation at different inter stimulus intervals (ISIs), the startle response of individual larvae was measured over 10 stimuli, and linear regression used to analyse the resultant data. Inducing a deliberate habituation using 1 second ISIs showed that control fish were capable of learned habituation (Linear regression: $r^2$ = 0.7882, F (1, 7) = 26.05, P = 0.0014). The responsiveness, but not strength, of the AER was significantly decreased with an ISI of 30 seconds over 10 stimuli. This was indicative of habituation (Figure 4.15). Intervals of 45 seconds or longer did not cause significant habituation (either in strength or responsiveness of AER; P >0.05; Figure 4.15). An ISI of 60 seconds (with a random variation up to 80 seconds) was selected for further work, as this did not cause habituation and also allowed for data collection to be streamlined. (Figures 4.15 and 4.16; Linear Regression: $r^2$ = 0.1179, F (1, 7) = 0.9359, P = 0.3656). Initial stimuli induced a consistently stronger AER than subsequent stimuli, as shown by the one sample t-test (Figure 4.16; t (8) = 13.68, P < 0.0001; t (8) = 26.46, P < 0.0001 for 1 and 60 second ISIs respectively), and was therefore removed from the datasets before performing regression tests (see Discussion Section of this Chapter). The number of stimuli chosen in the final startle protocol was 8. This number of stimuli allowed for a good range of frequencies (to provide a good picture of functional damage) and also prevented the possibility that habituation would occur.

### 4.12.5 Outcome

By optimising for the factors described above (Section 4.12), an average control compliance of 49.2% was obtained. This percentage value was determined by taking an average of all the percentage values for startle compliance over all of the final compound trials.

## 4.13 Plate design in the startle assay

In the initial test run of the assay (using the positive control neomycin), a computer program was used to randomly allocate treatment group. The treatments were assigned by column and included: one negative (solvent/E3 alone) control group, a range of four compound concentrations (up to the concentration required to damage all hair cells using the DASPEI assay (MTC HC)) and a positive control group. Following the initial test, it became apparent that it would be difficult to isolate the timing of the startle response for each stimulus based on a single control group. The reason for this was that the high-speed AER is probabilistic, giving only ~50% response, therefore it is more difficult to identify the response within a 24 well plate (as only two animals across the entire plate could be exhibiting a startle response). As a solution to this problem, the plate loading template was modified to include two additional control columns. The new plate design included: three control groups, one $IC_{50}$ concentration group, a top concentration group and a positive control. A comparison of the original plate design (neomycin test trial) and the optimised version (neomycin trial a) showed that including three control columns per plate gave a significantly stronger whole-plate AER than a single control column (Figure 4.17 A; 1.17 ± 0.14 compared with 0.62 ± 0.23 mm $s^{-1}$ respectively; *Mann Whitney U Statistic* = 97, 1 *d.f.*, $P < 0.0001$). In the majority of cases, the onset of AER was considerably easier to identify when three control columns were included (Figure 4.17 B-D).

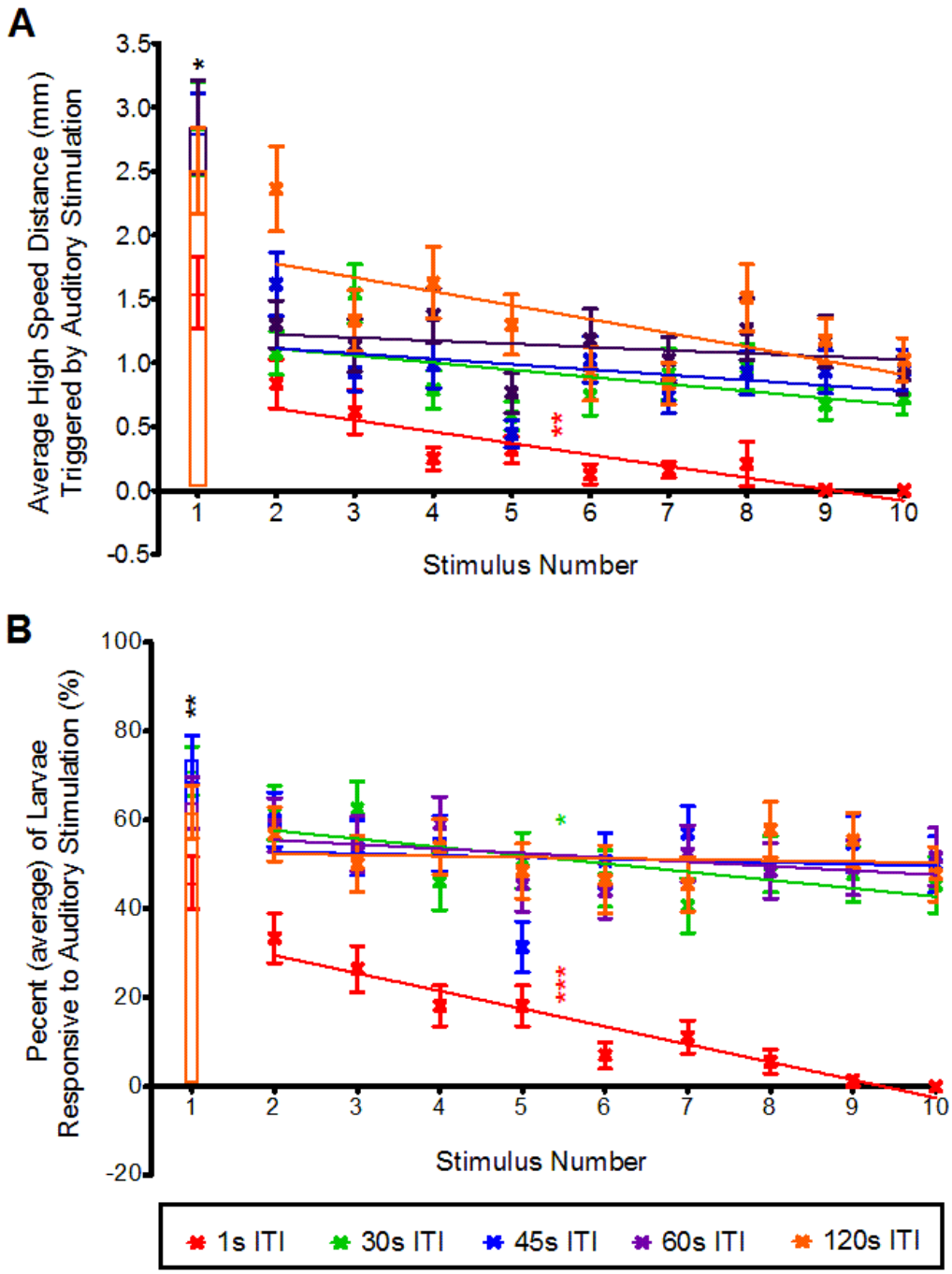



**Figure 4.15: The habituation profile of larvae over 10 repeated stimuli using ISIs of 1 s, 30 s, 45s, 60 s and 120 s.** (A) Auditory Evoked Responses of control animals at 200 Hz. An ISI of 1 s caused significant habituation. (B) Percent responsiveness of control larvae at 200 Hz. An ISI of 1 s or 30 s caused significant habitation. Each data point represents the average from 3 trials. The error bars show SEM.

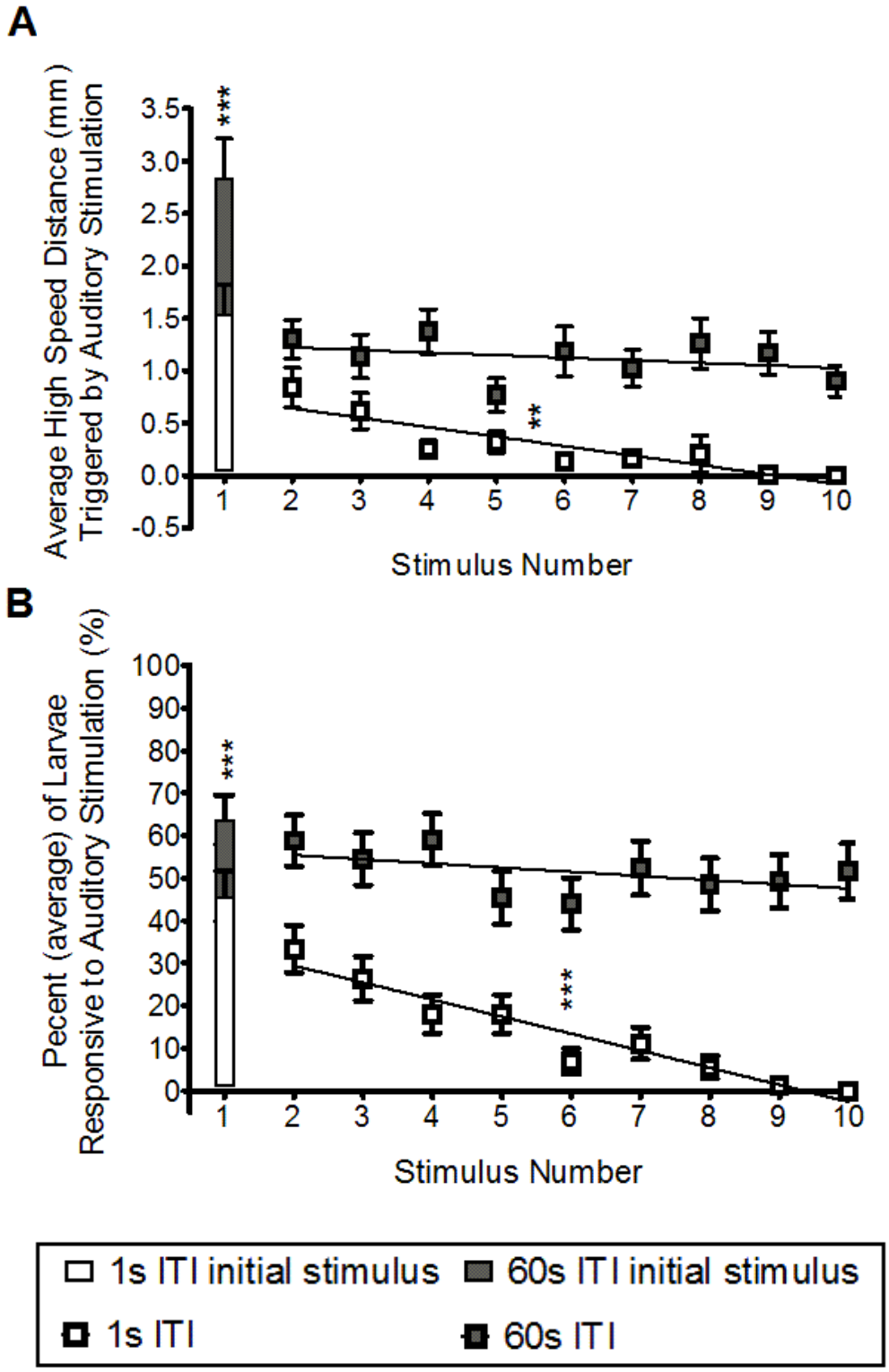



**Figure 4.16:The habituation profile of control larvae using the chosen 60 s ISI.** (A) Auditory Evoked Responses of control animals at 200 Hz. (B) Percent responsiveness of control larvae at 200 Hz. An ISI of 1 s caused significant habitation for both strength of startle (A) and responsiveness to stimuli (B). An ISI of 60 s did not cause habituation. Each data point represents the average from 3 trials. The error bars show SEM.

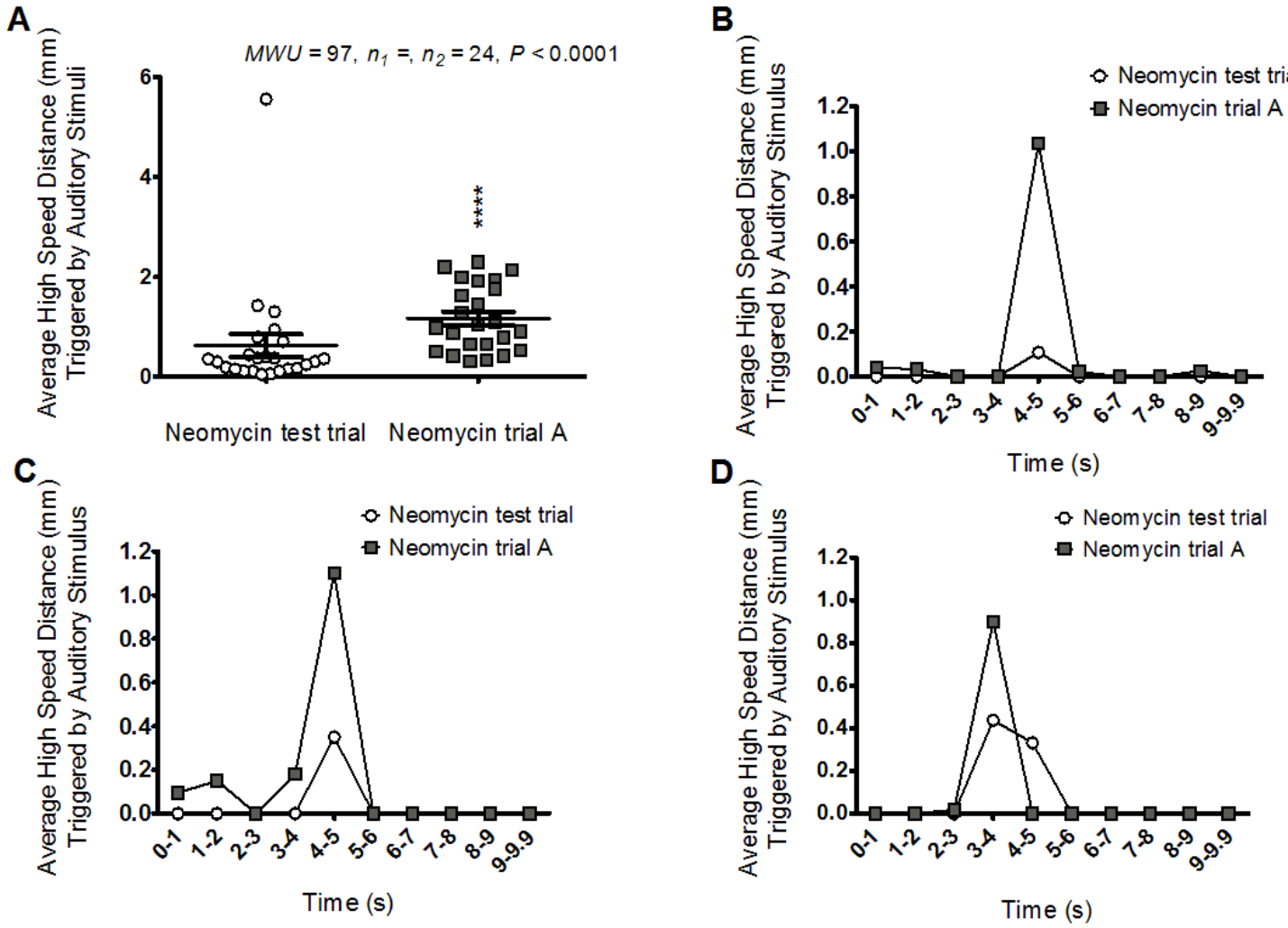



**Figure 4.17: The use of three control columns per 24 well microplate enables the identification of the onset of AERs.** (A) A comparison of "neomycin test trial" (one group of control animals only) and "neomycin trial A" (three control groups). Including three control columns per plate leads to a significantly stronger AER across the entire plate compared to a single control column. (B-D) Example graphs showing the whole plate response in the original test trial compared to the response in trial A. The AER onset is easier to identify for neomycin trial A. Data taken from: neomycin test trial and trial A; plates one (B), two (C) and three (D), (all at 200 Hz). (A) Individual data points show the peak 'average large distance' over a 10 second recording from 24 individual stimuli. Error bars represent SEM.

For the final assay, a pre-defined randomised control column for each of the three plates (control group 3) was used. By including a single control column only, it was possible to compare data with equal *n* numbers across the treatment groups. This was the most statistically valid and unbiased method of data analysis. It was important to show that by choosing control group 3, bias was not introduced into the assay. To analyse this, the data from neomycin trials A to C, for each control group (1, 2 and 3), were compared. There was no significant difference in the strength of the startle response between any of the control groups (Figure 4.18; one-way ANOVA followed by Bonferroni's MC test; $F$ (2, 105) = 0.2429, $P$ = 0.7848). This suggested that it was acceptable to use control group 3 only for the purpose of data analysis.

## 4.14 Deciding whether to merge time points

Occasionally, the larval startle response would straddle two of the 1 s time bins in the VideoTrack file making the response more difficult to pick out. Using only the maximum peak in these cases could have meant discarding useful data or missing the correct startle response altogether. To test this, the effect of merging versus not merging the two time peaks for neomycin trials A to C was compared. Time points were only merged when the secondary peak contributed substantially to the primary peak (i.e. when the smaller peak was at least 40% of the larger peak. See Figure 4.19 A). Merging of the time points had no significant effect on the outcome of the startle assay (Figure 4.19 B; two-way ANOVA; $F$ (1, 274) = 6.268 x $10^{-5}$, $P$ = 0.9937). It was decided that it would be more time-effective not to merge the time points prior to data analysis.

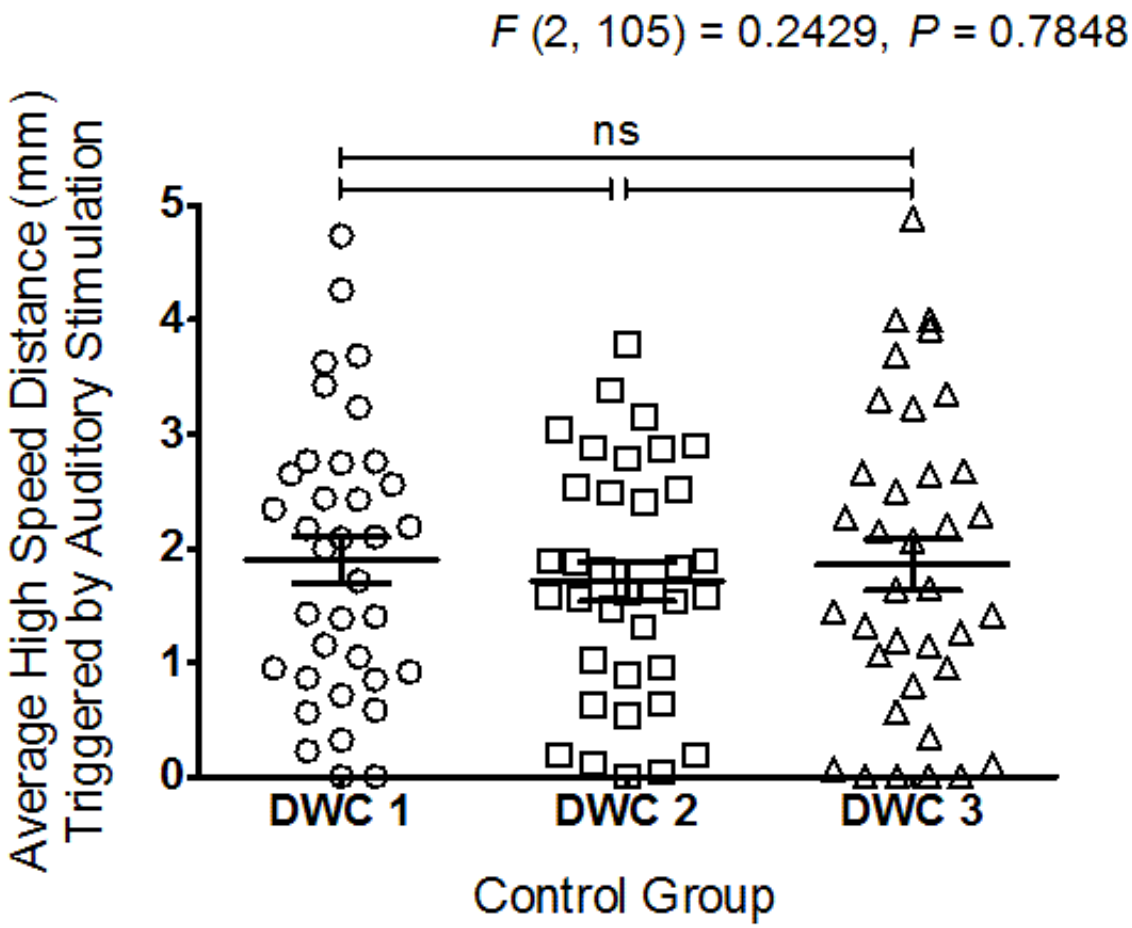



**Figure 4.18: The use of control column three only to analyse larval AERs.** There is no significant difference in startle responses between the three control groups, as shown by one-way ANOVA and Bonferroni's MC test. Using only control column three for statistical testing of larval AERs is a valid method and should not introduce bias. Data points analysed are from neomycin trials A-C (n = 12 per treatment group, per trial). Error bars represent SEM.

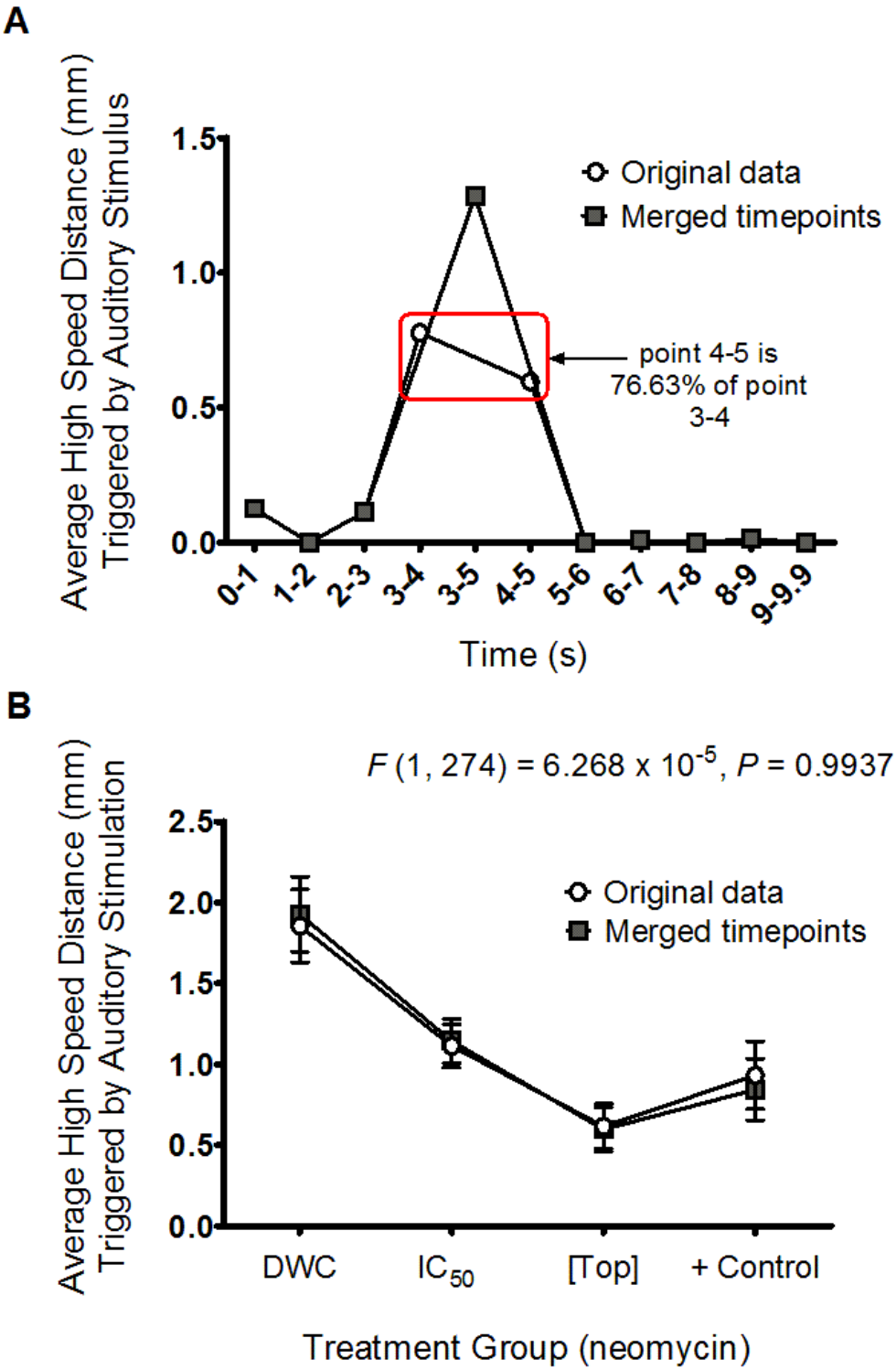



**Figure 4.19: Merging of time points does not affect the outcome of the startle assay.** Occasionally an AER would occur over two of the 1 s time bins (A). Time points were merged only when the smaller of the two peaks was at least 40% of the primary peak. Data are taken from neomycin trial A plate 1 40 Hz. (B) A comparison of merging versus non-merging of time points for neomycin trials A-C. Merging of time points had no significant effect on the outcome of the startle assay (two-way ANOVA followed by Bonferroni's MC test). Data points shown in (B) are the average of 3 trials (n of 12 per treatment group per trial). Error bars represent SEM.

## 4.15 The final startle assay

### 4.15.1 Standard Operating Procedure

A standard operating procedure was designed, based on the results of the optimisation of the assay. For full details of the protocol see Chapter 2, Section 2.9.6 and Appendix 2.

### 4.15.2 Macros

Macros were designed in Microsoft Excel in order to process the high volume of data effectively (see Appendix 1 and Supplementary Material disk for original macro files).

## 4.16 Investigating the effects of circadian rhythm on larval activity

In light of evidence that circadian rhythm could affect movement, it was necessary to examine the effect of time of day on baseline activity and startle reactions (Burgess and Granato, 2008; Cahill et al., 1998; Hurd et al., 1998).

### 4.16.1 Effects on baseline activity

Figure 4.20 shows the data obtained from testing the effects of time of day on baseline activity. For comparisons, two averages were used: total distance travelled over the 60 s recording period (Figure 4.20 A, C), and the average speed of larvae during the period in which they were detected (by tracking software; Figure 4.20 B, D). When data were compared for all three control groups (AM versus PM), time of day had a significant effect on baseline activity (Figure 4.20 A, B). Larvae travelled further (230.4 ± 7.086 compared with 208.8 ± 6.923 mm respectively; *Mann Whitney U Statistic* = 7816, $n_1 = n_2 = 138$, $P = 0.0101$) and faster (4.021 ± 0.1223 compared with 3.66 ± 0.1101 mm $s^{-1}$ respectively; $t = 2.195$, 276 *d.f.*, $P = 0.029$) in the morning than in the afternoon. When data were compared only for control group three (the control group used in the final assay to statistically analyse data), time of day did not affect baseline activity (Figure 4.20 C, D). There was no significant difference in average distance travelled (214.4 ± 14.07 compared with 207.3 ± 13.15 mm respectively; $t = 0.3656$, 87 *d.f.*, $P = 0.7155$) or speed (3.843 ± 0.2556 compared with 3.588 ± 0.2152 mm $s^{-1}$ respectively; $t = 0.7585$, 87 *d.f.*, $P = 0.4502$) between the morning and the afternoon.

### 4.16.2 Effects on startle response

Figure 4.21 shows the data obtained from testing the effects of time of day on startle response. The average high speed AER was used for analysis. When data were compared for all three control groups (AM versus PM), time of day had no significant effect on startle response (Figure 4.21 A; 1.794 ± 0.1078 versus 1.980 ± 0.1127 mm; *Mann Whitney U Statistic* = 9475, $n_1$ = 143, $n_2$ = 142, $P$ = 0.3296). Additionally, there was no significant difference in average high speed AER between the morning and the afternoon when control groups when examined individually (Figure 4.21 B-C).

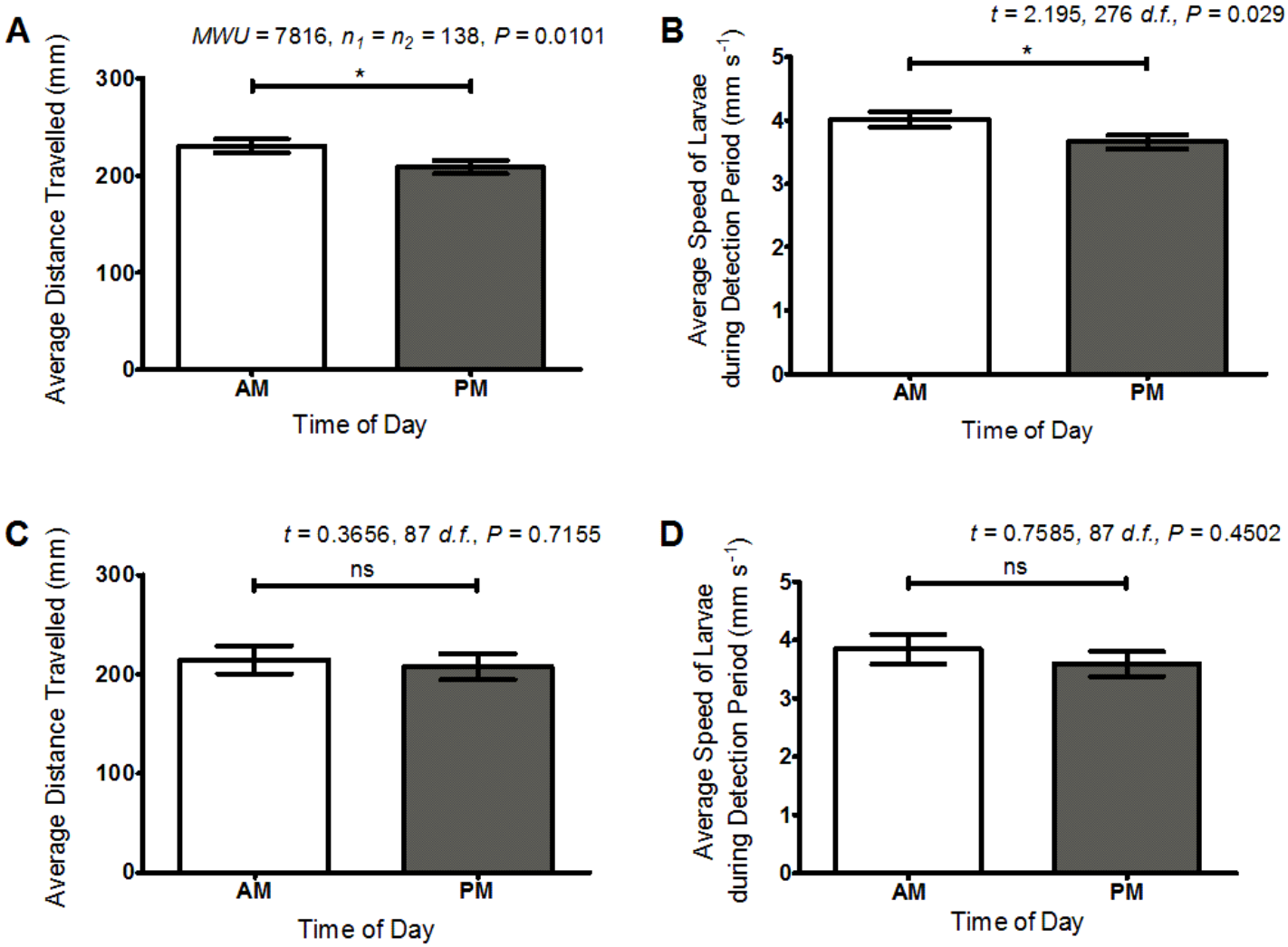



**Figure 4.20:The effects of time of day on baseline activity of animals.** (A) The overall distance travelled in a 60 s video recording for all three control groups (morning versus afternoon). (B) The average speed of larvae during the period of detection for all three control groups (morning versus afternoon). In a comparison including all three control groups, the baseline activity of larvae is significantly reduced in the afternoon. (C) The overall distance travelled in a 60 s video recording for control group three only (morning versus afternoon). (D) The average speed of larvae during the period of detection for control group three only (morning versus afternoon). In a comparison of control group three only, baseline activity is unaffected by time of day. All data are represented as mean average values, error bars are the SEM.

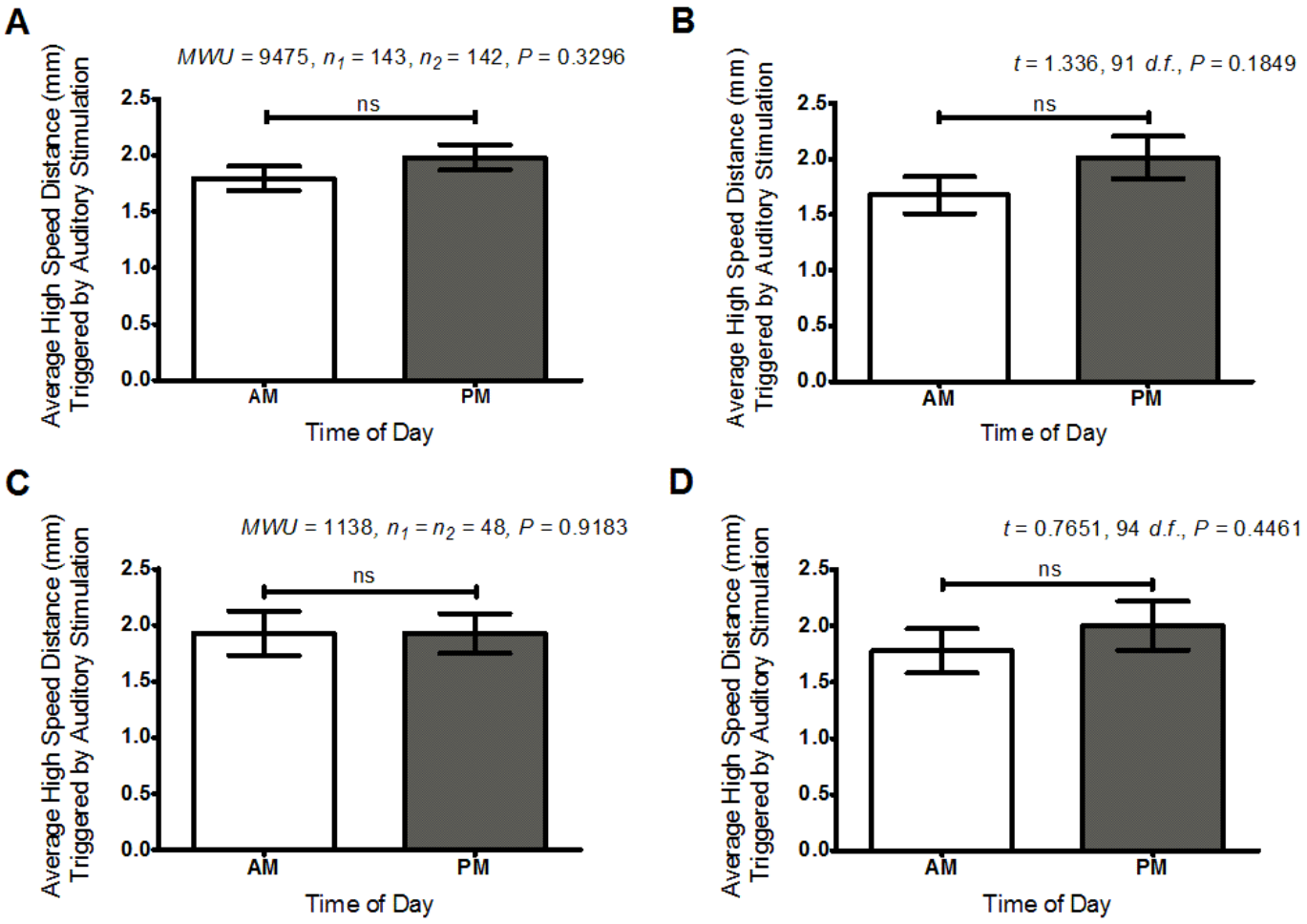



**Figure 4.21: The effects of time of day on startle activity.** (A) The average high speed distance travelled in response to auditory stimulation for all three control groups (morning versus afternoon). (B) The average high speed distance travelled in response to auditory stimulation for control group one (morning versus afternoon). (C) The average high speed distance travelled in response to auditory stimulation for control group two (morning versus afternoon). (D) The average high speed distance travelled in response to auditory stimulation for control group three (morning versus afternoon). (A-D) Time of day does not appear to affect the noise-evoked startle response in control animals. All data are represented as mean average values, error bars are the SEM.

# Discussion

## 4.17 The assessment of Larval Startle was constrained by the limitations of the equipment

### 4.17.1 Speaker

The range of frequencies that could be used to elicit the startle response in larvae was limited by the size of the speaker. The large 15” speaker used in the assay had a frequency range of 35 Hz to 4 kHz; this range could theoretically assess the response of the lateral line (frequencies up to ~ 200 Hz) and the ear (frequencies above ~ 200 Hz) to auditory stimulation. Frequency range was further limited by the output of the amplifier and the response of larval fish to the sinusoid. The large speaker was chosen with this in mind. There was a balance between obtaining an even spread of signal across a large diameter and obtaining frequencies within the higher range. Although it would be feasible to optimise the assay for an additional small speaker capable of vibrating at higher frequencies, this would only provide information concerning the ability of larvae to respond to higher frequencies and not further information about ototoxic effects. Other assays have shown that larval zebrafish can respond to frequencies of up to 1200 Hz using broadband vibrational stimuli created by a shaker (Burgess and Granato, 2007; Burgess et al., 2009; Zeddies and Fay, 2005).

### 4.17.2 Lighting

Problems with lighting conditions in the laboratory meant that an on/off flicker could be seen on playback of video recordings. As a consequence, it was necessary to reduce the frame rate of the camera. By improving the lighting, the frame rate could have been increased, enabling the in-depth kinematic type analysis of the startle response (Burgess and Granato, 2007; Fontaine et al., 2008). A way of solving this problem would have been to custom make and fit an infrared lighting system similar to the one used in the Viewpoint Inc. ZebraBox; this would have been designed in the shape of a ring and positioned around the camera, preventing shadows from the camera obscuring the view of the plate. Despite the decreased frame rate, it was still possible to carry out valuable analysis of the zebrafish response to acoustic stimulation and to distinguish between larval startle response and general high-speed movements

on playback. The tracking used was a somewhat more sophisticated method of analysis than the frame subtraction method used by others, which can only really detect if any movement has occurred (Bang et al., 2002; Zeddies and Fay, 2005).

### 4.17.3 On/Off click at high frequencies

An audible on/off click was encountered at the higher frequencies (above 800 Hz) due to the requirement to drive the stimulus at higher amplitudes. In order to fix this problem it was necessary to add a switch into the circuit between the amplifier and speaker that limited the stimulus duration to less than ~ 100 ms. The modification was unsuccessful in trials and was therefore not adopted, leading to the removal of higher frequencies from the assay.

### 4.17.4 Extraneous noise

As far as possible, extraneous noise was excluded from the assay during the equilibration and assay periods. Unfortunately, the equipment that maintained the laboratory at 28ºC also made a constant gentle humming noise. The only sure way of eliminating extraneous noise would have been to carry out all of the experiments in an anechoic chamber.

### 4.17.5 Triggering

In order to streamline the video recording and analysis of the startle response, attempts were made to use a trigger that would activate the camera recording at the same time as the stimulus onset. However, the trigger did not work for technical reasons and as such all recordings were carried out manually. This meant that the AER did not always take place at exactly the same time in all of the video recordings. In order to overcome this problem, a macro was designed in Microsoft Excel that could take the raw data and automatically determine when the startle response was occurring. This method was 100% accurate in the example recordings and provided an adequate alternative to triggering the recording.

## 4.18 Multiple types of larval startle were observed

As described in Section 4.2, there are alternative types of startle reflex. When the video recordings were reviewed by eye, both C- and S-starts (Kimmel et al., 1974; Liu et al., 2012) were observed. When the videos were examined in more

detail, it was possible to pick out both short and long latency C-starts (SLC and LLC respectively). Responses were classified manually: S- and C-starts were coded as “S”/”SE” while LLCs were coded “MS”, “LS” or “VMS”. The observation of all of these types of escape response is suggestive that the optimised stimuli can elicit the full array of noise evoked responses. All types of startle were included in the final dataset where possible. Occasionally, due to the threshold setting procedure, some mild startles (LLCs) may have gone undetected.

## 4.19 Determining the startle threshold of control larvae required compromise

### 4.19.1 Detection threshold for individual larvae

Setting the threshold for the detection of individual animals was an important process in ensuring accurate tracking of the larvae. This was because the algorithms for detection of startle and baseline movement were dependent upon detection of the animal in each well plate. The threshold for animal detection is set when an adequate number of pixels are displayed over each fish; these pixels define the centre of gravity. The threshold for larval detection was set to 115; this was the best possible threshold to detect the larvae over the 20 sample videos. Occasionally, this general threshold setting meant that some fish were not detected by the software, even though they were clearly moving. As a consequence, some fish were inaccurately tracked in baseline activity and startle experiments (example seen in Figure 4.8 B). This was mainly a problem when assessing baseline activity. To account for this, baseline activity was displayed in two ways 1) the total distance moved during the recording 2) the total distance moved divided by the amount of time detected by the tracking software (not the total time of the recording).

### 4.19.2 Startle threshold

The threshold chosen to define a high-speed AER was 21.1 mm $s^{-1}$. This threshold struck a good balance between stringency and detection of startle, showing a good concurrence between the Viewpoint traces and the scoring by eye. However, even with this optimised threshold, there were still discrepancies between automated detection and scoring by eye. There were a number of explanations for this. The most common explanation was that it was difficult to

separate a mild startle from a fast movement. Another frequently encountered reason for a mismatch was that larvae were difficult to track at the edges of wells. Less often, fish were unresponsive to the stimulus but performed an unrelated movement within the 1 s tracking time. Very occasionally, fish went undetected due to the software or startled with a duration of less than 100 ms and were tracked in the “small distance” category (a limitation of the software). Although the threshold for the startle was a compromise, the data obtained by tracking does closely match the scoring by eye and is adequate for a medium throughput design. To further refine the assay, more thresholds could be tested between 21.1 and 26.6 mm $s^{-1}$. Interestingly, 21.1 mm $s^{-1}$ is similar to the threshold set to detect hyperactivity in seizure liability testing (>20 mm $s^{-1}$), although the movement patterns are very different (single bend versus continuous high-speed circling of the well; Winter et al., 2008).

## 4.20 Control compliance of larvae in the startle assay was dependent on many factors, including raising density and inter stimulus interval

A major obstacle in examining larval AERs is that short latency C-starts are probabilistic (Burgess and Granato, 2007). In the current investigation, early testing showed that only 40% of untreated larvae responded to each stimulus. A number of parameters were altered in order to increase the control compliance of animals, thus optimising the assay. In particular, raising density, ISI duration and plate type were found to be key determinants of control compliance. Refining these factors resulted in a final control compliance overall of 49.2%.

Previous work has shown that larval husbandry conditions can have a direct effect on animal sensitivity to auditory stimuli. Larvae raised at lower densities show increased responsiveness (Burgess and Granato, 2008). The data presented here fit with this, and suggest that AER is significantly greater in animals raised under low density conditions. Larval development appeared to be unaffected in the large sample of animals tested, meaning that the observed effect is probably not explained by detectable alterations in development. More likely, larvae raised at a higher density will be exposed more often to vibrational stimuli through the water, due to a higher number of surrounding larvae performing swim bursts. This higher background level of underwater motion and

therefore increased stimulation may desensitise the larval zebrafish to subsequent artificial stimuli (Domenici, 2010).

It is well recognized that repeated exposure to a stimulus results in a gradual decline in response (Rankin et al., 2009; Thompson and Spencer, 1966). This form of non-associative learning is called habituation. Larval zebrafish are capable of habituation to repeated stimuli, including tone bursts. In support of this, deliberate induction of habituation was shown here using an inter-stimulus interval (ISI) of 1 second. To determine the duration between stimuli that could prevent habituation, the larval response to different ISIs was investigated. The current data showed that movement elicited by the first auditory stimulus is significantly greater than that brought about by successive stimuli; this is in agreement with a previous study of habituation in larval zebrafish (Best et al., 2008). This result justified the removal of the first stimulus from further testing, as the immediate alteration in response between stimulus one and two is not classed as a habituation effect. Experimental data showed that habituation did not occur over repeated stimuli with an ISI of 45 seconds or longer; this is in agreement with work by Zeddies and Fay (2005) who used an ISI of 105 ± 30 seconds to prevent a decrease in response to stimulation. Previous studies have used an ISI of 15-20 seconds, which was shown to be insufficient to induce habituation (Best et al., 2008; Burgess et al., 2009). In contrast, the data generated here show that an ISI of 30s can reduce the responsiveness of the larvae over ten repeated stimuli. These minor discrepancies in larval habituation are most likely due to inter-strain variation and different experimental methodology between studies. I selected an ISI of 60 seconds with a random variation of up to 20 seconds to prevent habituation. This 60 second gap also streamlined the experimental protocol, allowing time for video processing to be carried out in between each stimulus.

In early pre-clinical screens, it is important to maximise the quality and throughput of assays by increasing sample size. I compared larval AERs between 24- and 48-well plates in order to test whether the capacity of the current assay could be increased by using more wells per experiment. Unfortunately, the data indicated that the best response could be observed using 24-well plates. This result limited the throughput of the assay and

introduced a necessity for staggered treatments to obtain an appropriate number of animals per treatment group. Importantly, using 24-well plates provided more robust data with lower variability overall.

## 4.21 Time of day did not affect baseline activity or larval startle in the optimised assay

Previous studies have suggested that time of day can have a significant effect on locomotor behaviours in larval zebrafish (Burgess and Granato, 2008; Cahill et al., 1998; Hurd et al., 1998). Although time of day had an effect on baseline activity over all three concentrations combined in the current study, it did not appear to affect control groups two or three individually. Control group three was used as the pre-determined control group to analyse the locomotor and startle activity of zebrafish in the assay. Time of day had no effect on startle response for any of the control groups, combined or taken individually. Taken together, these data suggested that there was no difference in behaviour between the morning and afternoon recordings. In reality, it is unlikely that a difference of approximately 4 hours or less during an artificial light period would have a large effect on activity. This result should be viewed with some caution however, as data were analysed retrospectively and also animals were at slightly different stages of development. Further analysis of animals would be necessary to confirm this finding.

## 4.22 Improving the design of the assay gave promising preliminary results

Additional control treatment columns were included in the plate design at the expense of a range of test compound concentrations. The preliminary results obtained from neomycin testing were improved by using this new plate design instead of the initial plate design.

By creating re-useable macros in Excel, it was possible to greatly reduce the time taken to process the high volume of data obtained through the visual tracking software for the neomycin test. These macros could be used to process the data obtained for each new compound in the main startle assay.

Designing a simple standard operating procedure and proforma (Appendix 2) for the startle assay ensured consistency in performing the experiments, making the results more reliable.

## 4.23 Concluding remarks

The startle assay was optimised for a number of conditions, ensuring the best possible responses in control larvae. The assay was pilot tested and the design of the assay was adjusted to ensure that the most accurate data could be obtained. Using this optimised assay it was possible to assess the effects of compounds of interest on larval auditory evoked responses. The chapter that follows summarises and discusses the results of the functional testing of larval AERs, rheotaxis and underwater motion detection following compound exposure.

# Chapter 5 The Effect of Ototoxins on the Function of the Lateral Line

## Introduction

### 5.1 Aim

The aim of the work described in this chapter was to exploit the optimised startle assay (Chapter 4), in combination with additional reflex-based assays, to investigate the functional consequences of exposure to selected ototoxins. Additional aims were to assess the relative sensitivities of these functional assays and to determine the suitability of the zebrafish to model hearing impairment using functional assessment.

### 5.2 Assays of zebrafish *acousticolateralis* function

#### 5.2.1 Startle

As described in Chapter 4, acoustically-evoked startle is an indicator of the function of the lateral line in zebrafish. The startle response is conserved in many higher order species including humans. In humans, the audiogenic startle reflex is characterised by eye closure, grimacing, neck flexion, trunk flexion, abduction of the arms, bending of the elbows and pronation of the forearms (Jacobson, 1926; as reviewed by Dreissen et al., 2012; Wilkins et al., 1986). In patients with hearing loss, the audiogenic startle is reduced or non-existent. This is also the case in other vertebrates such as rodents and mice (Carlson and Willott, 1996; Horlington, 1968; Jaspers et al., 1993; Koch, 1999; Willott et al., 1998). A principle aim of the current study was to investigate whether the startle reflex in fish was reduced in response to hair cell damage, thus mimicking the result in humans and other higher vertebrates. The hypothesis was that test substances that caused a significant increase in hair cell damage, (as indicated by reduced DASPEI fluorescence) would also reduce the response of larval zebrafish to sinusoidal tone bursts over a range of frequencies, alongside other indicators of response impairment.

#### 5.2.2 Rheotaxis

The ability of larvae to orientate to, and swim against, currents is termed rheotaxis (Arnold, 1974). This behaviour is conserved in many fish species and

can be observed as early as 5 dpf in free-swimming zebrafish larvae (Baker and Montgomery, 1999a; Baker and Montgomery, 1999b; Baker and Montgomery, 2001; Coombs et al., 2001; Montgomery et al., 1995; Olszewski et al., 2012; Suli et al., 2012); it plays an important role in natural behaviours such as station holding (holding position in flowing water), breeding and feeding (Arnold and Weihs, 1978; Montgomery et al., 1995). Historically, it was proposed that the visual and tactile systems were the key mediators of rheotaxis but it is now known that the hair cells of the mechanosensory lateral line are also key mediators of the behaviour in zebrafish (Johnson et al., 2007; Olszewski et al., 2012; Suli et al., 2012) and other fish species. A number of studies in adult fish have shown that ablating the hair cells of the lateral line can decrease the rheotactic ability of the animal (Baker and Montgomery, 1999b; Montgomery et al., 1997), however little is known about the effect of such damage on rheotaxis in larvae. Here, rheotactic behaviour in response to artificial circular currents was examined in larvae treated with ototoxins. It was hypothesised that lateral line-specific hair cell damage induced by ototoxins would lead to a reduced rheotaxis score.

### 5.2.3 Motion detection

The lateral line is capable of detecting subtle changes in water flow created by both stationary and moving objects, sensing “touch at a distance” (Dijkgraaf, 1963; Montgomery et al., 1995). Consequently, the seeker response test has previously been used to assess the responsiveness of animals following exposure to compounds that may reduce sensitivity to stimulation (e.g. hypnotics and sedatives, see Winter et al., 2008). In the context of the current study, the seeker response (SR) test served to answer two questions: are larvae responsive to water flow disturbances? And; do they retain neuromuscular function after compound exposure? A previous study using a flow stimulus to induce startle responses has shown that neomycin treatment causes a decreased responsiveness to water flow (McHenry et al., 2009). Therefore, the hypothesis was that treatment with ototoxins would result in a reduced seeker response score and that negative compounds would have no discernible effect.

## Methods

The techniques described in Chapter 2 were used to perform the assays to detect the functional consequences of exposure to test compounds. For full methods, see Sections 2.4.4, 2.9.6 to 2.11 and 2.13.

## Results

## 5.3 Assessment of the larval startle response after ototoxin exposure

The startle assay measured the larval high-speed response to a set of eight individual auditory stimuli. The standard response in control animals is a high-speed escape, characterised by whole body bending and burst movement (AER).

### 5.3.1 The outcome of treatment with positive test compounds

Results from the startle assay are shown in Figure 5.1. As predicted, larvae treated with neomycin trisulphate, streptomycin sulphate, gentamicin sulphate and aspirin displayed a significantly reduced high-speed escape response to auditory stimulation. For all the compound treatments except gentamicin sulphate, the reduction in startle response could only be detected at concentrations that damaged nearly all the hair cells; these concentrations were based on data from the DASPEI fluorescence assay. At the estimated $IC_{50}$ level for neomycin, streptomycin and aspirin (50% reduction in fluorescence score) there was no significant decrease in auditory evoked startle, despite an apparent qualitative reduction in larval responsiveness.

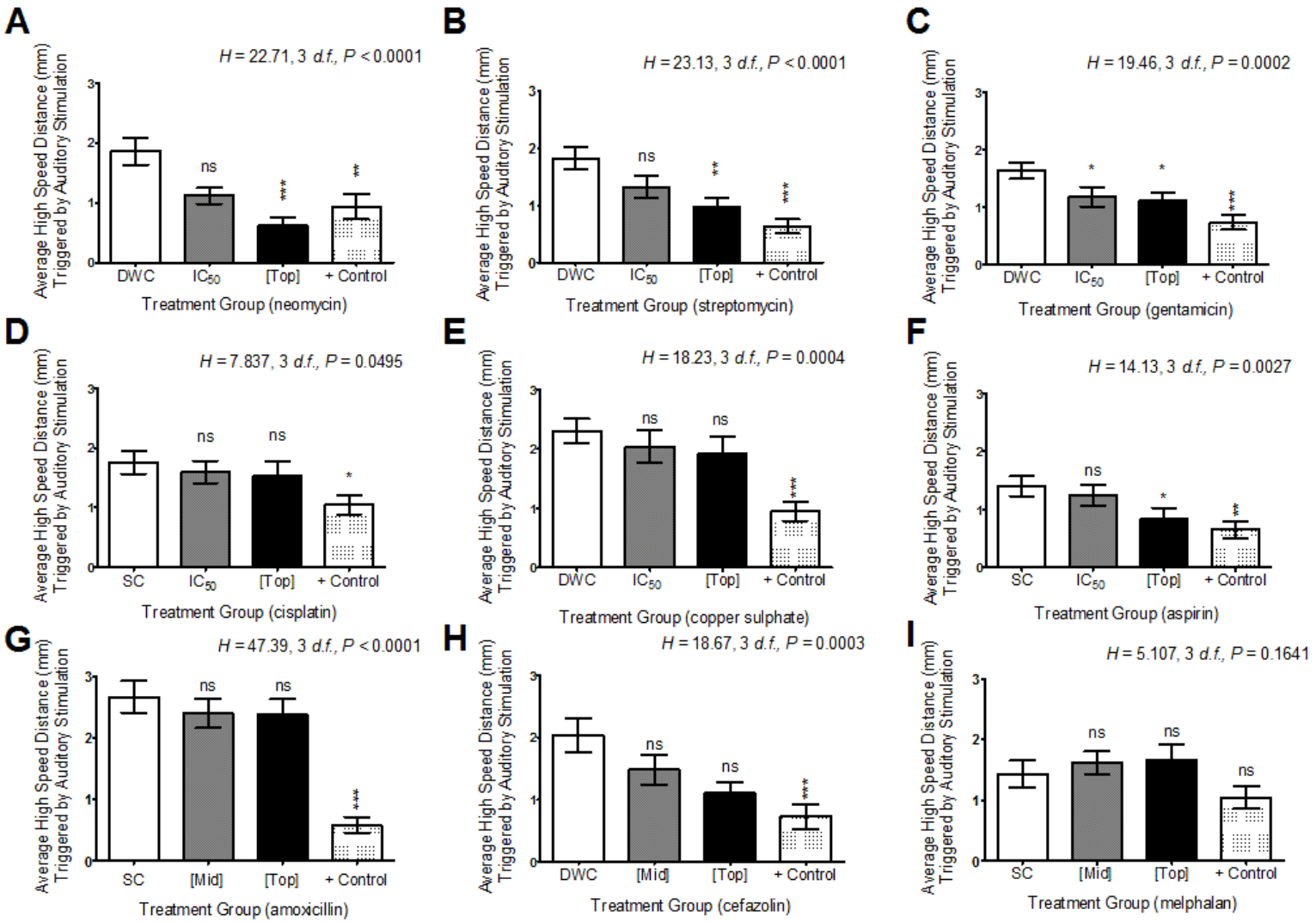



**Figure 5.1: Certain compounds caused a concentration-dependent decrease in larval startle response following histological damage.** Panels (A) to (I) are for different compounds. Neomycin (100µM ) was the positive control used. Minimum of 36 larvae pooled from 3 experimental repeats per condition. All data were analysed using the Kruskal-Wallis test, followed by Dunn's multiple comparison test. Statistical significance compared with the control group is indicated by asterisks. For details of compound exposures, see Table 2.1.

It was hypothesised that all compounds that caused a concentration-dependent reduction in DASPEI fluorescence in the pLL would also significantly reduce the larval AER. Contrary to this, larvae exposed to cisplatin and copper sulphate did not display a significantly decreased AER at the concentrations required to cause maximal loss of fluorescence in the DASPEI assay (Figure 5.1 D and E). In both cases, a mild but non-significant decrease in response was observed at the top concentration (cisplatin = 13.2% decrease, copper sulphate = 16.6% decrease). This "false negative" result suggested that the hair cells of the pLL were not fully damaged by the treatment, despite small the decrease in fluorescence.

### 5.3.2 Investigating hair cell integrity in compounds that gave a false negative result

#### 5.3.2.1 Cisplatin

One explanation for the unexpected negative result for cisplatin is that during the course of the study, two different samples of the compound were used. Both samples of cisplatin were of the same product code and specification from Sigma-Aldrich, although by necessity were from two separate batches. Batch 029K1426 was used at the University of Sheffield for the hair cell assay, but could not be reordered for use in the functional assessment at AstraZeneca, Brixham. When the hair cell assay was repeated, the second batch of cisplatin (069K1236) showed variable results: a higher concentration was required to reduce the DASPEI staining in the majority of the hair cells, shifting the concentration response curve to the right (Figure 5.2) compared to batch 029K1426. The estimated $IC_{50}$ for batch 029K1426 was 14.5 µM (95% confidence level = 8.2 µM to 25.5 µM) compared to 143.2 µM (95% confidence level = 88.8 µM to 231.0 µM) for batch 069K1236. It is possible that the AER of larvae would have been significantly affected at a higher concentration of batch 069K1236 of cisplatin, as more of the cells would have been damaged.

#### 5.3.2.2 Copper sulphate

As an alternative assessment of hair cell integrity following exposure to copper sulphate, the transgenic line *Tg(pou4f3::mGFP)s356t* was used to examine hair cell GFP expression. In transgenic larvae treated with 1 µM copper sulphate, GFP expression was unaltered in pLL hair cells up to one hour post-treatment, despite a reduction in live hair cell labelling with DASPEI and FM1-43FX. However, neomycin treatment at the top concentration reduced GFP expression in the posterior lateral line (refer to Chapter 3 Figures 3.7 and 3.18). The retention of GFP-positive hair cells in the lateral line of larvae exposed to copper could indicate that some hair cell functionality remained, accounting for the unaltered AER. GFP expression in the *Tg(pou4f3::mGFP)* line is an additional indicator of hair cell viability, along with DASPEI or FM1-43FX staining, which appeared more sensitive to minor hair cell damage.

### 5.3.3 The role of the ear in maintenance of the startle response following hair cell damage to the lateral line

Treatment at the highest concentrations of all the ototoxins never completely abolished the larval AER (Figure 5.1), suggesting that some other sensory component may also contribute to the startle response. This additional sensory component is most likely the ear. In support, the inner ear hair cells of the transgenic *Tg(pou4f3::mGFP)* line retained a high level of GFP expression and the correct morphology after acute exposure to the ototoxins by immersion, even when GFP expression in the pLL was reduced or eradicated (Figure 3.18). It is probable that the ototoxins were unable to enter the inner ear, and thus inner ear hair cells were protected from damage. These observations indicate that the ear may play a role in mediating the larval startle response.

### 5.3.4 The outcome of treatment with negative control compounds

As expected, treatment with the negative control compounds amoxicillin, cefazolin and melphalan had no significant effect on the response of animals to auditory stimulation (Figure 5.1, G-I). This supported the results of the DASPEI assay that showed staining in the lateral line was unaffected by immersion in solutions of these compounds.

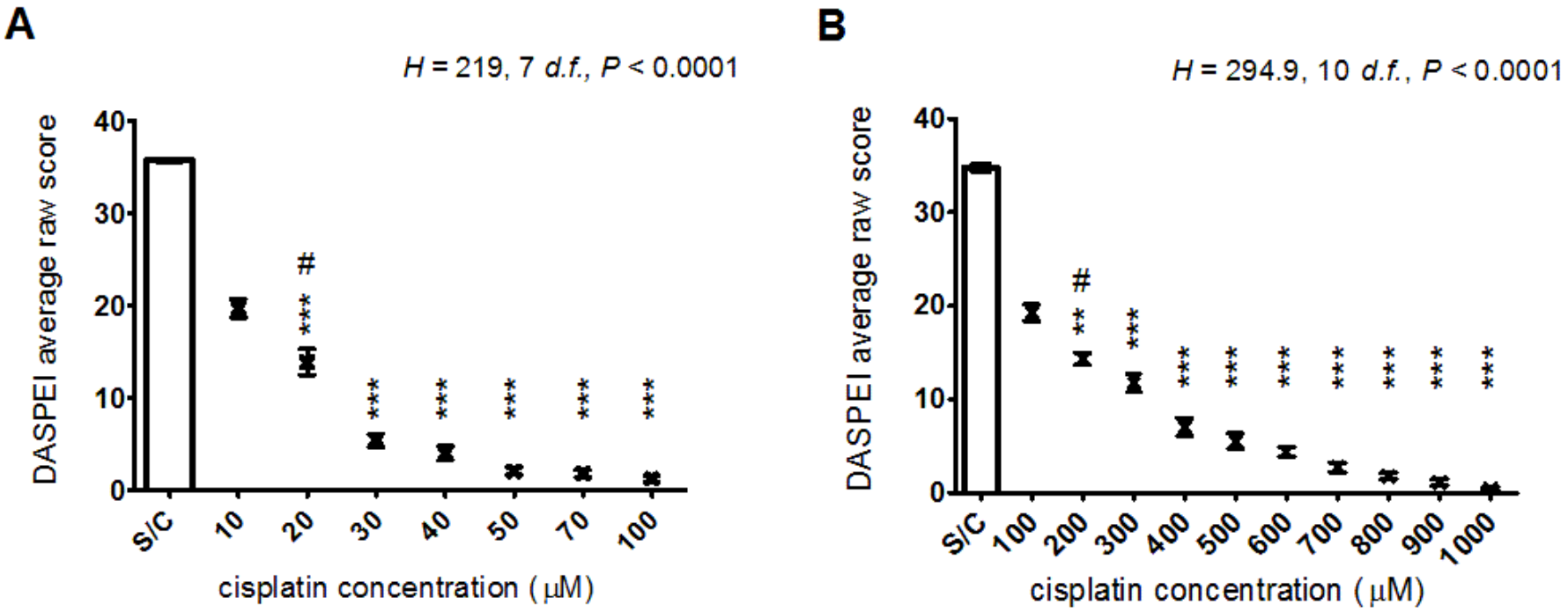



**Figure 5.2: Treatment of larvae with different batches of cisplatin shifts the concentration response curve and $IC_{50}$ value.** (A) Larvae treated with batch 029K1426. The hair cell MTC is approximately 100 µM and the estimated $IC_{50}$ is 14.5 µM. (B) Larvae treated with batch 069K1236. The hair cell MTC is approximately 1 mM and the estimated $IC_{50}$ is 143.2 µM. Data are pooled from three repeats for each compound, minimum *n* of 36 per treatment group. Data were analysed using the Kruskal-Wallis test, followed by Dunn's multiple comparison test. Statistical significance compared with the control group is indicated by asterisks.

### 5.3.5 The effects of compounds over individual frequencies

The results from the startle assay displayed in Figure 5.1 show the average AER of each of the larvae over all of the frequencies combined. The responses of larvae at individual frequencies were examined in order to probe the frequency sensitivity of the lateral line. It was hypothesised that the decrease in AER would be more apparent at lower frequencies where there was less contribution from the inner ear. In theory, damage solely to the lateral line could act to separate the roles of the lateral line and ear in frequency discrimination. The results for neomycin are as shown in Figure 5.3.

At most frequencies, a significant decrease in AER was observed at the top concentration of neomycin but not at the $IC_{50}$ level (Table 5.1). There was no change in AER at either treatment level compared to the solvent controls at 40 Hz and 100 Hz. This result was surprising, as it was expected that the functional damage to the lateral line would be more evident below 200 Hz. The best frequency for detection of functional damage in neomycin treated larvae was 200 Hz. At this frequency, a significant decrease in AER was seen at the $IC_{50}$ and at the top concentration (Kruskal-Wallis test followed by Dunn's multiple comparison test; $H$ = 12.63, 2 *d.f.*, $P$ = 0.0018).

In all other compound treatments, the individual frequency response to auditory stimulation was inconsistent (Table 5.1). For example, with streptomycin treatment significant alterations in AER were observed at the top-concentration level at 100 Hz and 200 Hz. For gentamicin, a reduction in AER was observed only at 50 Hz with the highest concentration of treatment. Aspirin treatment at 300 µM resulted in a decrease in AER at 40 Hz and 200 Hz. From these data combined, there was no clear pattern to indicate which frequency was most affected by exposure to ototoxins in general (i.e. lateral line hair cell damage). For the assay to indentify an individual ototoxin successfully, the frequencies had to be pooled together, rather than examined individually. By combining the data, it was possible to look at the *overall* change in response to auditory stimulation elicited by each compound.

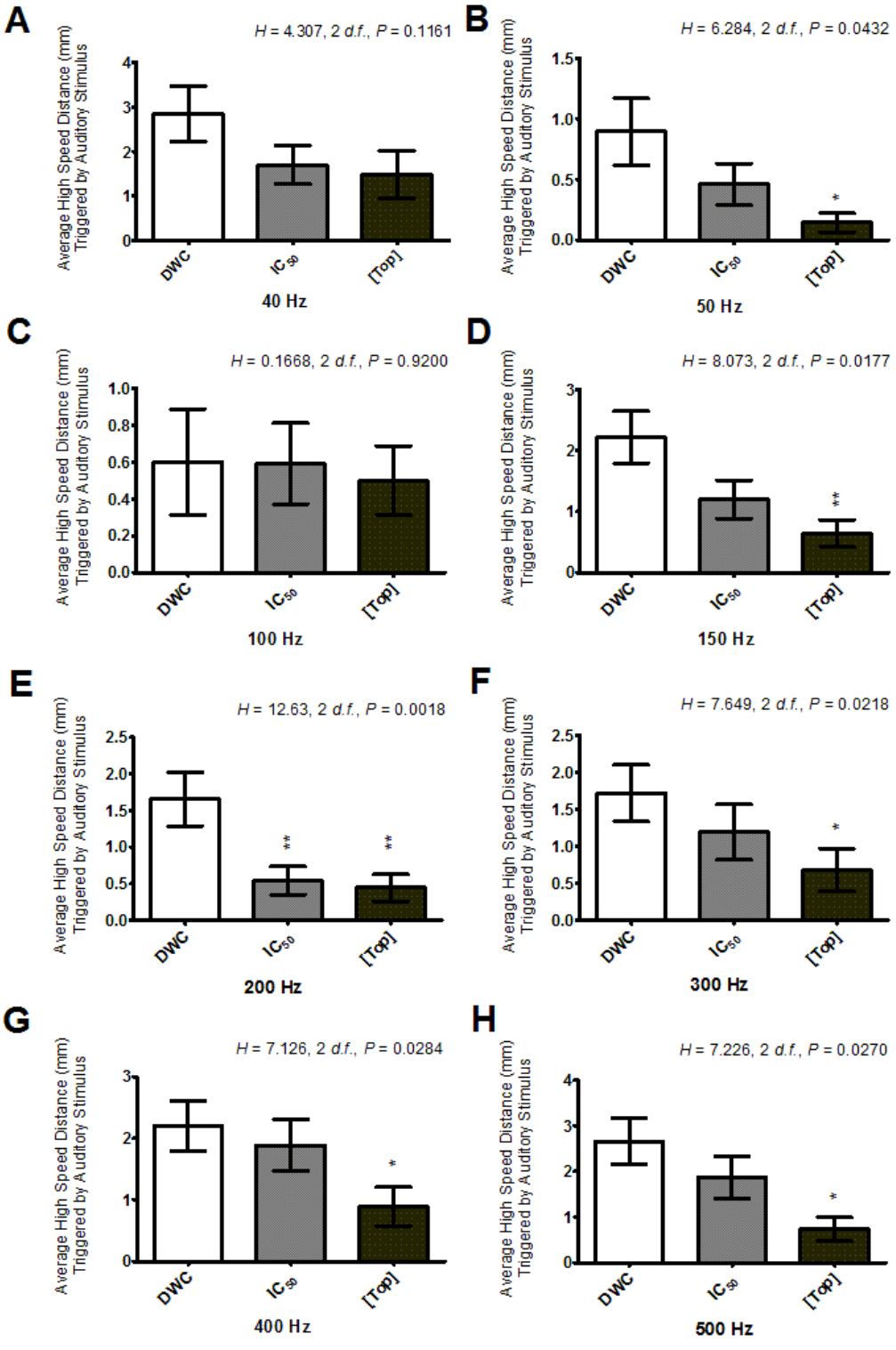



**Figure 5.3: The response of neomycin treated larvae to auditory stimulation at different frequencies.** Data were pooled over three repeats. A minimum 36 larvae per treatment group were tested. Data were analysed using the Kruskal-Wallis test, followed by Dunn's multiple comparison test. Statistical significance compared with the control group is indicated by asterisks. The stimulus frequency increases from panels (A) to (H).

| Test Substance | Frequency (Hz) | Control vs. $IC_{50}$ | Control vs. Top concentration |
|---|---|---|---|
| Neomycin | 40 | ns | ns |
| | 50 | ns | * |
| | 100 | ns | ns |
| | 150 | ns | ** |
| | 200 | ** | ** |
| | 300 | ns | * |
| | 400 | ns | * |
| | 500 | ns | * |
| Streptomycin | 40 | ns | ns |
| | 50 | ns | ns |
| | 100 | ns | * |
| | 150 | ns | ns |
| | 200 | ns | *** |
| | 300 | ns | ns |
| | 400 | ns | ns |
| | 500 | ns | ns |
| Gentamicin | 40 | ns | ns |
| | 50 | ns | * |
| | 100 | ns | ns |
| | 150 | ns | ns |
| | 200 | ns | ns |
| | 300 | ns | ns |
| | 400 | ns | ns |
| | 500 | ns | ns |
| Cisplatin | 40 | ns | ns |
| | 50 | ns | ns |
| | 100 | ns | ns |
| | 150 | ns | ns |
| | 200 | ns | ns |
| | 300 | ns | ns |
| | 400 | ns | ns |
| | 500 | ns | ns |
| Aspirin | 40 | ns | * |
| | 50 | ns | ns |
| | 100 | ns | ns |
| | 150 | ns | ns |
| | 200 | ns | * |
| | 300 | ns | ns |
| | 400 | ns | ns |
| | 500 | ns | ns |
| Copper Sulphate | 40 | ns | ns |
| | 50 | * | ns |
| | 100 | ns | ns |
| | 150 | ns | ** |
| | 200 | ns | ns |
| | 300 | ns | ns |
| | 400 | ns | ns |
| | 500 | ns | ns |

**Table 5.1: The effects of ototoxins at individual test frequencies.** Minimum of 36 larvae per treatment group and frequency. Significance is denoted by asterisks.

## 5.4 Can a reduction in AER due to ototoxins be rescued by increasing stimulus volume?

In mammalian studies, a widely used functional readout of hearing loss (and otoprotection) is the hearing threshold change. Consequently, the usefulness of the startle assay in measuring threshold changes in larval zebrafish was assessed in the current study. Results of the preliminary experiment in control and neomycin treated larvae are shown in Figure 5.4. Linear regression was used to analyse the effects of increasing stimulus volume on the larval AER. In control animals, increasing the volume of the stimulus from -30 dB TP to -15 dB TP had no significant effect on the larval AER (linear regression; $r^2$ = 0.0011, $F$(1, 275) = 0.3058, $P$ = 0.5807). In larvae treated with 100 µM neomycin, where the startle response was significantly attenuated, increasing the stimulus volume resulted in a significant rescue of AER (linear regression; $r^2$ = 0.01573, $F$(1, 257) = 4.107, $P$ = 0.0437). This rescue was especially marked at the highest volume (-15 dB TP) where the result for both treatment groups overlapped (4.117 ± 0.46 for controls and 3.870 ± 0.64 for neomycin treated animals). Thus, neomycin treatment increased the threshold for AER.

## 5.5 Baseline recording

Baseline recordings were analysed to ensure that the alterations in AER that were observed were not due to neuromuscular stimulant or sedative effects of the compounds.

### 5.5.1 Increased activity

As summarised in Table 5.2, treatment with amoxicillin caused a significant increase in the average total distance travelled during the recording period, compared to the control group (one-way ANOVA; $F$ (2, 95) = 4.914, $P$ = 0.0093). Acute exposure to amoxicillin caused a significant increase in average total movement from 156.6 ± 9.85 mm to 195.8 ± 8.94 mm; this increase was observed only at the mid concentration but not at the top concentration. Despite this, there was no overall change in activity observed for speed (during the period of larval detection; Kruskal-Wallis test; $H$ = 4.977, 2 *d.f.*, $P$ = 0.0831), suggesting the observed increase was artifactual, rather than biologically significant. For all other compounds, there was no significant increase in baseline activity between control and treated groups.

### 5.5.2 Decreased activity

There was no significant decrease in either the average speed of treated larvae over the time detected or the average total distance moved when the majority of compounds were compared to control groups (Table 5.2). Treatment with neomycin, however, coincided with a significant decrease in overall activity during detection time (Kruskal-Wallis test; $H$ = 15.83, 2 *d.f.*, $P$ = 0.0004) and in the average distance travelled (Kruskal-Wallis test; $H$ = 15.92, 2 *d.f.*, $P$ = 0.0003). Acute exposure to 100 µM neomycin resulted in a significant decrease in average speed compared to untreated larvae (Dunn's multiple comparison test; 2.119 ± 0.23 compared with 3.467 ± 0.20 $mmsec^{-1}$ respectively; $P < 0.05$) and in total distance moved (Dunn's multiple comparison test; 104.3 ± 13.34 compared with 191.8 ± 13.77 respectively; $P < 0.05$), suggesting some impairment of locomotor activity.

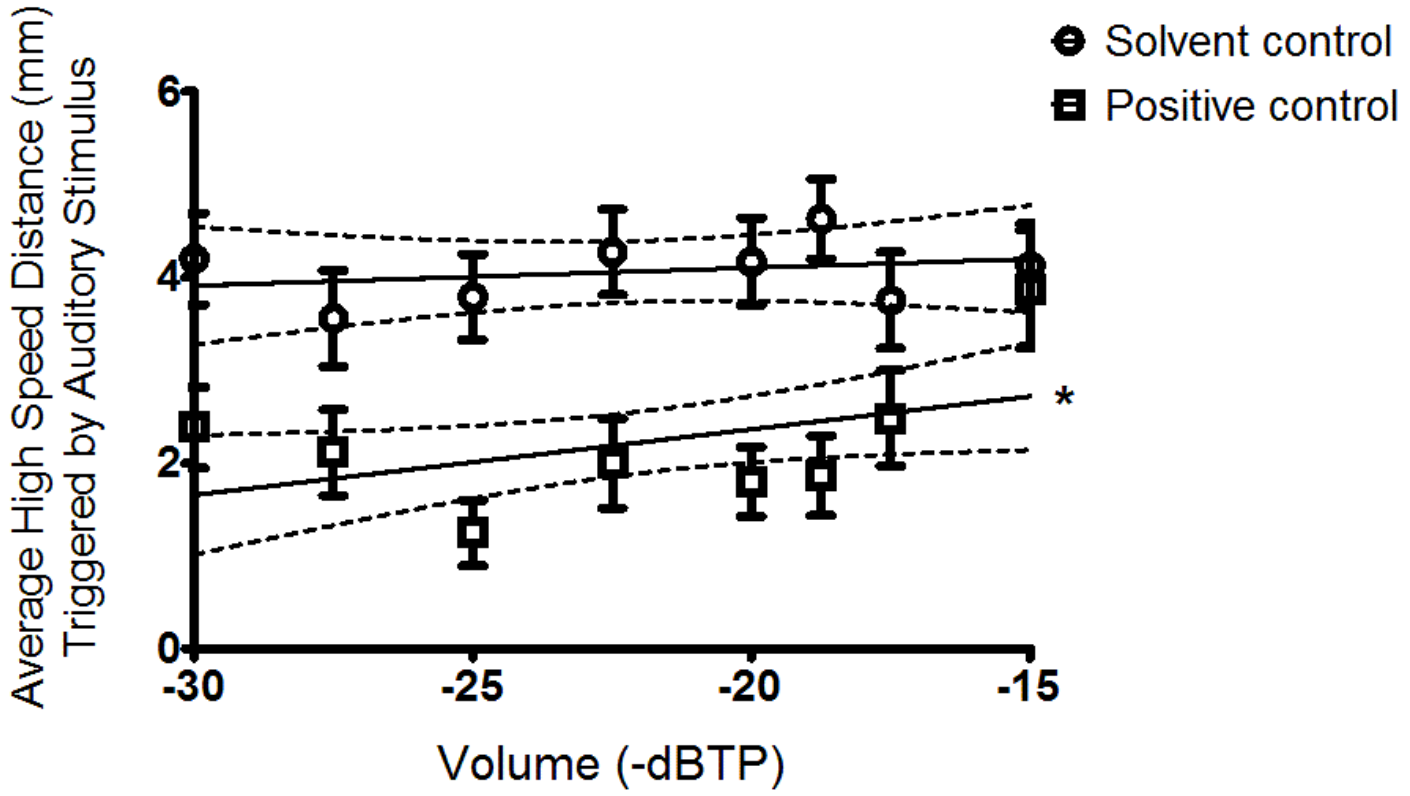



**Figure 5.4: The threshold response of larvae to auditory stimulation is shifted by neomycin treatment.** The response of larvae is significantly rescued by increasing the volume of the stimulus. Data were analysed using linear regression. Data were pooled from three experimental repeats. A minimum 36 larvae per treatment group were tested for each frequency.

| Test substance | Significant change in baseline activity over time detected? | Statistical Data (ANOVA/Kruskal-Wallis) | Concentration required to significantly increase/ decrease activity level? | Significant change in total distance travelled? | Statistical Data (ANOVA/Kruskal-Wallis) | Concentration required to significantly increase/ decrease activity level? |
|---|---|---|---|---|---|---|
| neomycin | Yes | $H$ = 15.83, 2 *d.f*., $P$ = 0.0004 | 100 µM ($P$ < 0.05, Dunn's post-test) | Yes | $H$ = 15.92, 2 *d.f*., $P$ = 0.0003 | 100 µM ($P$ < 0.05, Dunn's post-test) |
| streptomycin | No | $F$(2,93) = 0.6848, $P$ = 0.5067 | | No | | |
| gentamicin | No | $H$ = 1.261, 2 *d.f.,* $P$ = 0.5324 | | No | | |
| cisplatin | Yes | $F$(2,100) = 4.231, $P$ = 0.0172 | None | Yes | $F$(2,100) = 5.255, $P$ = 0.0068 | None |
| aspirin | No | $F$(2,94) = 1.655, $P$ = 0.1966 | | No | | |
| copper sulphate | No | $F$(2,94) = 1.825, P = 0.1669 | | No | | |
| amoxicillin | No | $H$ = 4.977, 2 d.f., P = 0.0831 | | Yes | $F$(2,95) = 4.914, $P$ = 0.0093 | Mid dose only (P < 0.05, Dunnett's post-test) |
| cefazolin | No | $F$(2,98) = 0.3355, $P$ = 0.7158 | | No | | |
| melphalan | No | $F$(2,98) = 0.6207, $P$ = 0.5397 | | No | | |

**Table 5.2: Movement profiles from drug-treated larvae identify potentially sedative effects of compounds.** Neomycin treatment reduced the average speed of unstimulated larvae. All other histologically positive and negative compounds had no significant reduction in average speed of the larvae over the time they were detected for. Amoxicillin at the $IC_{50}$ level caused an increase in total distance moved (mm). Minimum of 36 larvae pooled from 3 experimental repeats per condition. Statistical tests performed were one-way ANOVA (followed by Dunnett's multiple comparison tests) or Kruskal-Wallis test (followed by Dunn's multiple comparison test).

To investigate further, neomycin was re-examined using the final startle protocol alongside two concentrations of MS222 anaesthetic (0.1 µg/mL or 0.0125 µg/mL). The purpose of the extra tests was to assess the possibility that neomycin had sedative effects. The higher concentration of anaesthetic induced a state of full sedation and non-responsiveness after only a few minutes. The lower concentration was insufficient to cause full sedation after a 30 minute exposure and caused only a mild decline in responsiveness and activity. The average speed of neomycin-treated larvae was significantly faster than heavily sedated larvae (Figure 5.5; 2.409 ± 0.27 $mmsec^{-1}$ versus 1.229 ± 0.21; $P$ <0.05), but no different from the lower concentration of MS222 (2.409 ± 0.27 versus 2.572 ± 0.19 $mmsec^{-1}$). The average total distance moved was significantly different between neomycin treatment and heavy sedation (132.7 ± 14.94 versus 55.56 ± 11.18; $P$ <0.05). There was no significant difference in average total distance moved between neomycin treatment and light sedation (132.7 ± 14.94 versus 148.8 ± 11.79; $P$ >0.05). This data suggested that the effects of 100 µM neomycin treatment were comparable to light, not heavy, sedation. Additional tests were carried out using rheotaxis and SR to identify any differences between neomycin treatment and light sedation groups (see Section 5.6.3).

## 5.6 Additional functional testing

### 5.6.1 Rheotaxis tests

As shown in Table 5.3, treatment with the aminoglycosides (neomycin, streptomycin and gentamicin), cisplatin and aspirin resulted in significant disruption to rheotactic behaviour. Treatment with neomycin and gentamicin at the $IC_{50}$ level was sufficient to decrease rheotaxis score (Bonferroni's multiple comparison test, $P < 0.01$; 22.2% reduction for neomycin, 15.3% reduction for gentamicin). In contrast, the highest concentration of compound was required to reduce rheotaxis score with streptomycin, aspirin and cisplatin treatment (Bonferroni's multiple comparison test, $P < 0.05$; 15.3% reduction for streptomycin, 21.3% reduction for aspirin, and 11.1% reduction for cisplatin). Interestingly, copper sulphate treatment did not significantly impair rheotactic behaviour at either concentration. At the concentration of copper sulphate required to decrease DASPEI fluorescence in majority of hair cells, there was an 18% decrease in rheotaxis but this did not achieve statistical significance. In the solvent and negative control treated groups, there was no change in rheotaxis score following treatment (Bonferroni's multiple comparison test, $P > 0.05$).

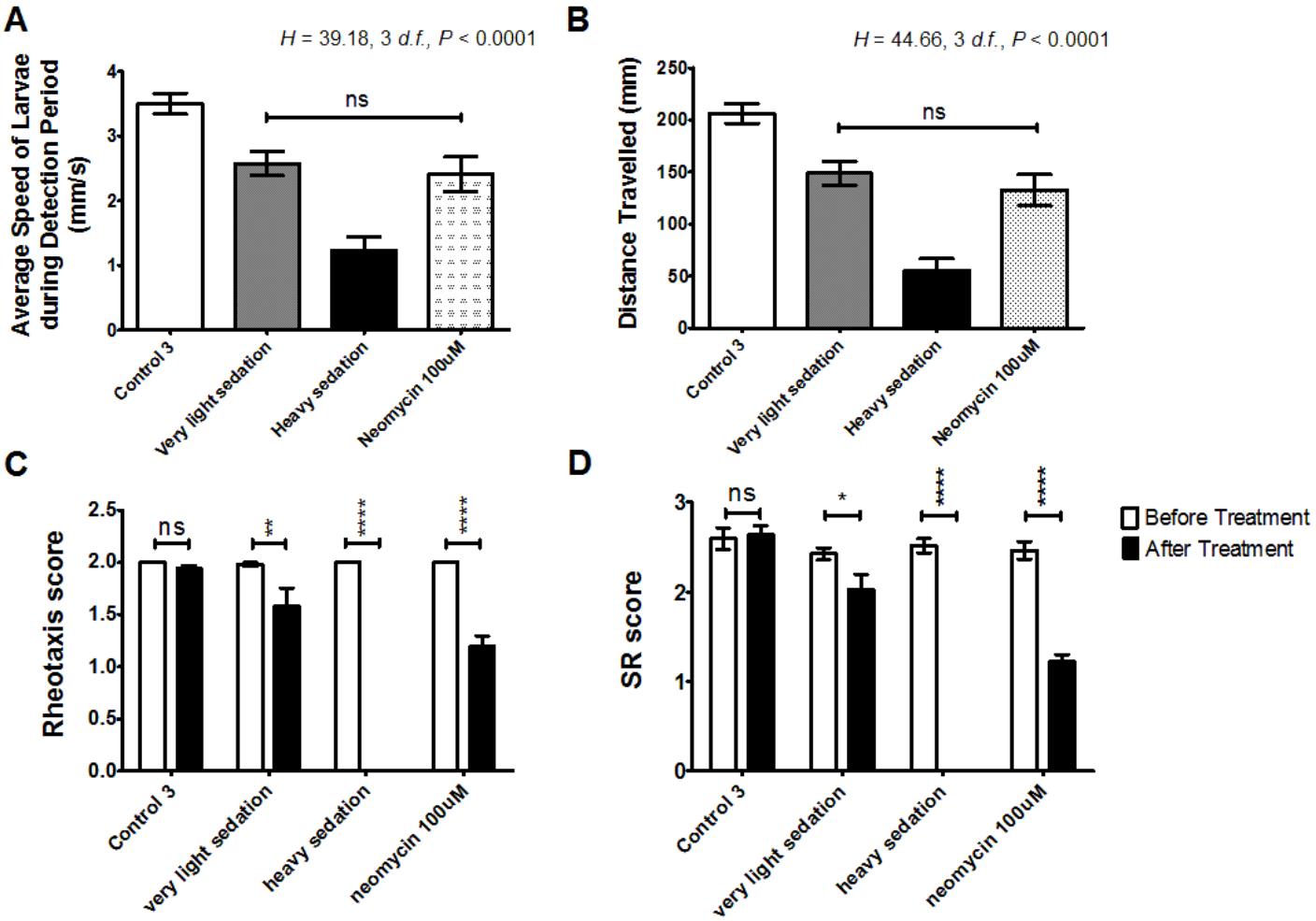



**Figure 5.5: The effects of neomycin versus MS222 on the movement profile, rheotaxis and SR score of larvae.** (A) and (B) Examination of the baseline movement profiles of larvae treated with top concentration neomycin or anaesthetic (analysed using Kruskal-Wallis test and Dunn's post-tests). (C) and (D) Rheotaxis and SR scores alter after immersion in neomycin or anaesthetic. Data from three experimental trials were analysed using Two-way ANOVA, followed by Bonferroni multiple comparison tests. Statistical significance level (α) is denoted by asterisks (*).

| Test Substance | Rheotaxis Score | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Percentage Change in Score After Compound Exposure and Significance Level of Change | | | | | | | |
| | DWC/SC | | $IC_{50}$/Mid-dose | | Top-dose | | + Control | |
| Neomycin | -0.4 | $t$=0.0673, ns | -22.2 | $t$=4.310, ** | -36.1 | $t$= 7.004, *** | -36.8 | $t$=7.071, *** |
| Streptomycin | 0 | $t$=0.0, ns | -6.9 | $t$=1.443, ns | -15.3 | $t$=3.175, * | -34.7 | $t$=7.217, *** |
| Gentamicin | -2.8 | $t$=0.686, ns | -15.3 | $t$=3.773, ** | -22.2 | $t$=5.488, *** | -26.4 | $t$=6.517, *** |
| Cisplatin | 0.5 | $t$=0.1240, ns | -5.6 | $t$=1.488, ns | -11.1 | $t$=2.977, * | -16.5 | $t$=4.341, ** |
| Aspirin | -1.4 | $t$=0.2073, ns | -15.3 | $t$=2.280, ns | -21.3 | $t$=3.178, * | -38.9 | $t$=5.803, *** |
| Copper Sulphate | -5.6 | $t$=0.8251, ns | -11.1 | $t$= 1.650, ns | -18.1 | $t$=2.682, ns | -30.6 | $t$=4.538, ** |
| Amoxicillin | -2.8 | $t$=0.9177, ns | -5.6 | $t$=1.835, ns | -5.6 | $t$=1.835, ns | -22.2 | $t$=7.341, *** |
| Cefazolin | 0 | $t$=0.0, ns | -2.3 | $t$=0.5692, ns | -1.6 | $t$=0.3997, ns | -45.3 | $t$=11.29, *** |
| Melphalan | -1.4 | $t$=0.2955, ns | -4.2 | $t$=0.8866, ns | 0 | $t$=0.0, ns | -29.5 | $t$= 6.287, *** |

**Table 5.3: Rheotaxis scores decreased in a concentration dependent manner with ototoxin exposure.** Data are presented as the percent reduction in score following treatment with either the water/solvent control, test compound or positive control for all test substances. Statistical data from post-tests are displayed in the adjacent column (for all post-test data, *d.f.* = 1). All data were analysed using two-way ANOVA, followed by Bonferroni multiple comparison tests. Minimum of 36 larvae pooled from 3 experimental repeats per condition. For details of compound exposure, see Table 2.1.

### 5.6.2 Seeker response tests

Results from the seeker response (SR) assay showed that all of the ototoxic compounds reduced the overall SR score by at least 46% at the top-concentration level. This suggested that the ototoxins caused a decrease in responsiveness to stimulation through the water in the larvae (summarised in Table 5.4). Larvae were still able to respond to direct touch in the SR assay after ototoxin exposure, indicating that neuromuscular function was not affected and so not the *ability* to respond. The negative controls, amoxicillin and cefazolin, had no significant effect on SR score. Melphalan treatment caused an unexpected 14% reduction in responsiveness at 400 µM (top-concentration level). In animals treated with solvent/water control there was no significant change in the seeker response score (Table 5.4).

### 5.6.3 Comparing rheotaxis and seeker response data in neomycin treated versus sedated animals.

To identify any subtle differences between 100 µM neomycin treatment and light sedation, the rheotaxis and seeker response scores of the animals were assayed (Figure 5.5). Following light sedation or treatment with the top concentration of neomycin there was a significant disruption to rheotactic behaviour (Figure 5.5 C). Interestingly, the effects of neomycin on this behaviour were more pronounced; rheotaxis score was reduced by 40.3% with neomycin compared to 20.2% for light sedation.

As shown in Figure 5.5 D, seeker response scores were significantly reduced for both treatment groups. A greater decline in responsiveness was observed with neomycin treatment than light sedation with MS222 (50.4% compared with 16.5%).

Taken together, these data show that 100 µM neomycin treatment is sufficient to induce a decline in movement comparable with very light sedation with MS222, but that neomycin exposure induces stronger effects on rheotaxis and SR overall. It is therefore possible that neomycin treatment (at the top-concentration level) causes a more complex behavioural effect than sedation and that sedation is not the sole reason for the observed reduction in AER.

## 5.7 Overall sensitivities of ototoxicity assays

A comparison of the data collected for the DASPEI, startle, rheotaxis and seeker response assays can be seen in Table 5.5. The sensitivity of each assay in detecting the ototoxic effect of each of the positive compounds is shown. The startle assay was the least sensitive assay overall, as it could only detect changes in response at the top-concentration level in 4 out of 6 of the compounds assessed. The most sensitive assays were the DASPEI and seeker response assays. In both of these assays, all of the positive compounds (6/6) were detected as ototoxins at the $IC_{50}$ level or lower. This result suggests that the startle assay is less sensitive in detecting drug-induced ototoxicity in larval zebrafish, and is probably more effective when used in conjunction with other assays for hearing impairment.

| Test Substance | Seeker Response Score | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Percentage Change in Score After Compound Exposure and Significance Level of Change | | | | | | | |
| | DWC/SC | | $IC_{50}$/Mid-dose | | Top-dose | | + Control | |
| Neomycin | 2.6 | $t$=0.4261, ns | -27.8 | $t$=4.497, ** | -61.6 | $t$=9.505, *** | -61.3 | $t$=9.634, *** |
| Streptomycin | 6.3 | $t$=1.449, ns | -35.6 | $t$=8.132, *** | -48.9 | $t$=11.11, *** | -61.2 | $t$=14.09, *** |
| Gentamicin | 4.4 | $t$=0.9912, ns | -40.2 | $t$=8.769, *** | -49.6 | $t$=10.60, *** | -59.5 | $t$=13.42, *** |
| Cisplatin | 8.7 | $t$=1.621, ns | -17.8 | $t$=3.511, * | -55.8 | $t$=10.98, *** | -51.4 | $t$=9.859, *** |
| Aspirin | 0.8 | $t$=0.1249, ns | -37.7 | $t$=5.725, *** | -45.8 | $t$=7.063, *** | -59.9 | $t$=9.337, *** |
| Copper Sulphate | 1.7 | $t$=0.2966, ns | -35.4 | $t$=5.753, *** | -46.9 | $t$=8.007, *** | -63.5 | $t$=11.45, *** |
| Amoxicillin | 9.0 | $t$=1.687, ns | -0.7 | $t$=0.1350, ns | 2.2 | $t$=0.405, ns | -60.6 | $t$=11.54, *** |
| Cefazolin | 3.5 | $t$=0.9288, ns | -8.4 | $t$=2.303, ns | -5.9 | $t$=1.613, ns | -62.8 | $t$=17.05, *** |
| Melphalan | -1.4 | $t$=0.3107, ns | -6.9 | $t$=1.553, ns | -13.7 | $t$=3.107, * | -58.2 | $t$= 13.28, *** |

**Table 5.4: Seeker response scores decreased in a concentration dependent manner with ototoxin exposure.** Data are presented as the percent reduction in score following treatment with either the water/solvent control, test compound or positive control for all test substances. Statistical data from post-tests are displayed in the adjacent column (for all post-test data, *d.f.* = 1). All data were analysed using two-way ANOVA, followed by Bonferroni multiple comparison tests. Minimum of 36 larvae pooled from 3 experimental repeats per condition. For details of compound exposure, see Table 2.1

| Test Substance | DASPEI Assay | | Startle Assay | | SR Assay | | Rheotaxis Assay | | Most Sensitive Assay? |
|---|---|---|---|---|---|---|---|---|---|
| | Lowest Detectable Concentration Effect (μM) | Significance *P* value | Lowest Detectable Concentration Effect (μM) | Significance *P* value | Lowest Detectable Concentration Effect (μM) | Significance *P* value | Lowest Detectable Concentration Effect (μM) | Significance *P* value | |
| Neomycin | 10 | * | 100 | * | 14 | ** | 14 | ** | DASPEI |
| Streptomycin | 10 | * | 200 | * | 40 | *** | 200 | * | DASPEI |
| Gentamicin | 50 | * | 25 | * | 25 | *** | 25 | ** | SR assay |
| Aspirin | 50 | * | 300 | * | 193 | *** | 300 | * | DASPEI |
| Cisplatin | 20 | * | - | ns | 14 | * | 100 | * | SR assay |
| Copper (II) sulphate | 0.5 | * | - | ns | 0.5 | *** | - | ns | SR assay |

**Table 5.5: The comparative sensitivities of the ototoxicity assays.** The various sensitivities are presented for each method. The most sensitive assay is decided first by lowest detectable concentration effect and then by significance level (α) at that concentration.

# Discussion

## 5.8 The startle assay is an indicator of the functional damage induced by ototoxins

The optimised startle assay was exploited to assess the effects of the chosen positive and negative compounds on the high-speed auditory evoked reflex. It was hypothesised that compounds that significantly decreased the DASPEI fluorescence score would also produce a functional effect; a measurable concentration-dependent decline in response to tone bursts over a range of frequencies. Data from the assay showed that certain ototoxins, including all of the aminoglycoside antibiotics and aspirin, caused a decrease in startle response at the maximum concentrations tested. The results of the assay agree with published data showing that other lateral line toxins, including cadmium and mercury, attenuate the C-start in other fish species (Baker and Montgomery, 2001; Faucher et al., 2006; Faucher et al., 2008; Weber, 2006). These data also concur with evidence from electrophysiological studies that auditory brainstem responses are reduced after gentamicin exposure in goldfish (Ramcharitar and Selckmann, 2010; Ramcharitar and Brack, 2010; Weber, 2006).

## 5.9 The startle assay lacks the sensitivity of the DASPEI assay in detecting ototoxicity

### 5.9.1 Detection rate for ototoxins

The startle assay was able to detect 67% of the positive compounds and 67% of negative compounds in testing. The positive control, neomycin, gave consistent results with a decrease in AER observed in 89% of trials. The startle assay was unsuccessful at consistently detecting decreases in AER at individual frequencies, but was a good predictor when all of the stimuli were combined to give a readout for each compound tested.

### 5.9.2 False negative results

The compounds cisplatin and copper sulphate were not shown to have any significant effect on response in the startle assay, despite giving a positive result in the DASPEI assay, thus giving a false negative result. The fact that the startle assay could not detect a decrease in AER for these compounds is probably explained by the high variability of response between test animals, and

possibly by incomplete hair cell damage, as well as batch differences between compounds. It is unlikely that the false negative results were caused by drug-induced activity changes leading to decreased responsiveness to stimulation, as there was no significant change in baseline activity of larvae exposed to either compound. Data from additional experiments suggests that some hair cell function may remain in the lateral line even in the absence of DASPEI staining and this could account, at least in part, for the false negative result. Perhaps differences in AER effects between aminoglycosides, cisplatin and copper sulphate could also be explained by compound-specific cellular damage mechanisms, although this would require further investigation.

### 5.9.3 Assay sensitivity

Overall, the startle assay lacked some sensitivity in comparison to the DASPEI assay: it was only sensitive to changes in AER at high concentrations that were sufficient to bring about near complete loss of staining in the DASPEI assay. There are a number of reasons why this might be the case. A simple reason for this could be that the behavioural response (AER) is highly variable in animals. The larvae react very differently to the same stimulus, leading to greater data variability. One method of making the results less variable would be to assign a rank score to different ranges of response based on the distance moved at high speed. This could be similar to the scoring system applied to the simpler functional assays (rheotaxis and SR). This rank scoring method has the potential to limit the variability of the data making it easier to detect any changes between treatment groups. However, it must be considered that ranking the data would also remove a large amount of quantitative information from the results, as the data would no longer be continuous.

Importantly, the sensitivity of the assay is somewhat limited because the measured response is probabilistic, even in control groups. This means that there is only approximately fifty percent response rate to each auditory stimulus at best. Additionally, the startle response is never completely abolished, presumably due to the contribution of the ear, making significant results harder to obtain. These factors together make any differences between groups more difficult to determine than if a higher compliance rate and a more marked decrease in AER were to be exhibited.

A final explanation for the low apparent sensitivity of the assay could be the stringency of the method for defining the stereotypical startle. In the finalised startle assay protocol, movements (“M”) below the high speed threshold were not recorded as AERs. The majority of movements below this threshold were characterised by a weak C-bend, but not the full range of motion expected from a stereotypical startle. The future inclusion of these less marked responses may be sufficient to increase the compliance levels in control larvae and thus make any compound-induced decreases in AER easier to detect.

### 5.9.4 Advantages of the startle assay

Despite its sensitivity problems, the startle assay has a number of advantages. The method used to test the compounds is medium throughput and high content, allowing for full analysis of each compound within approximately 18 hours (including MTC, DASPEI, startle, baseline activity, rheotaxis and seeker response assays). The startle protocol also has the potential to be adapted for use in combined endpoint testing alongside other behavioural assays, for example, sleep/wake cycle and seizure studies that all rely on high speed video analysis and subsequent behavioural phenotyping (Rihel et al., 2010; Rihel and Schier, 2012; Winter et al., 2008). Finally, the startle assay is not limited by one of the main potential complications of the use of larval zebrafish in early toxicology screens: a reliance on compound uptake across extraneous body surfaces. Insufficient uptake of test compounds in other larval zebrafish assays can result in erroneously low potencies for certain compounds when based on exposure concentration (Redfern et al., 2008; Richards et al., 2008; Winter et al., 2008). In the current assay, this problem is circumvented as the hair cells are essentially directly exposed to the concentration of compound dissolved in the solution.

### 5.9.5 Measuring threshold changes in AER using the startle assay

A promising outcome of preliminary testing was that neomycin exposure raised the volume threshold for auditory evoked responses. Thus, louder sound volume was able to rescue the startle response back to a level that was comparable to control animals. This mimics the type of result seen in mammalian studies using ototoxins (e.g. Ciarimboli et al., 2010). By using a

“threshold change” assay in future experiments, it may be possible to look more closely at the damage induced by each ototoxin and to investigate the rescue effects of otoprotectants. One caveat of this experiment is that the threshold of non-distortion of the sine wave is exceeded at the higher volume outputs. This means that the results are not reflective of pure tone audiometry and data may be affected by a reaction to the lower frequency harmonics of the sound wave.

## 5.10 The ear may play a role in larval startle

It has previously been shown that acute exposure of zebrafish adults and larvae to ototoxins causes damage specific to the lateral line which does not affect the ear (Blaxter and Fuiman, 1989; Matsuura et al., 1971). Despite a significant reduction in startle response with the aminoglycosides and aspirin at the top-concentration level, the reaction was never completely abolished. On further inspection using the transgenic line *Tg(pou4f3::mGFP)s356t*, it was found that hair cells in the inner ear were unaffected by immersion of the larva in ototoxins, whereas the hair cells of the lateral line displayed obvious damage. These findings are especially interesting and support the notion that the ear plays a role in initiating the noise-evoked startle response. This would also support the previous implication by Zeddies and Fay (2005) that the sacculus partly mediates the startle response. Further investigation will be necessary to separate out the roles of the ear versus the lateral line for this behaviour. For example, the injection protocol described in Chapter 3 could be used in conjunction with ototoxin exposure in order to assess the additive effects of hair cell damage to the inner ear on AER.

## 5.11 Rheotaxis and Seeker Response assays offer sensitive readouts of ototoxin-induced functional damage

### 5.11.1 Rheotaxis

As an additional test of lateral line function, rheotactic behaviour in response to artificial circular currents was examined. The rheotactic responses were compared in larvae before and after ototoxin treatment. It is known that the mechanosensory lateral line is a key mediator of rheotaxis in zebrafish (Johnson et al., 2007) and other fish species (Montgomery et al., 1997). It was hypothesised that lateral line-specific hair cell damage induced by ototoxins would lead to a reduction in rheotaxis score and that this change in behaviour

would not be seen in solvent control and negative control-treated larvae. The data generated here supported this hypothesis on the whole, with 83% of the positive ototoxic test compounds causing a reduction in rheotaxis score. All negative and solvent control treatments had no effect on rheotaxis. This is in agreement with data from other fish species, such as whitebait and blind cave fish, in which exposure to certain ototoxins and heavy metals is sufficient to raise the threshold for rheotactic responses (Baker and Montgomery, 2001; Montgomery et al., 1997). Since the publication of this work (Buck et al., 2012) there have been two new papers investigating the contribution of the lateral line system to rheotaxis. In one paper, the larval response to flow was investigated using a closed flow system in which the effects of neomycin-induced hair cell damage and EDTA treatment on rheotaxis were assessed. Neomycin treatment was found to significantly reduce percentage rheotaxis in the absence of visual cues. Disruption of mechanotransduction in the lateral line hair cells with EDTA treatment was sufficient to attenuate rheotaxis (Suli et al., 2012). In the second paper, an assay was developed which could measure lateral line mediated rheotactic burst responses. This study found that ablation of the lateral line hair cells using neomycin significantly reduced the rheotactic burst response (Olszewski et al., 2012). The experimental findings of both of these papers are in agreement with the data presented here, lending support to the results.

One unexpected result was that copper sulphate did not affect rheotaxis in the current assay, as this effect has previously been observed in larval zebrafish in response to copper exposure (Johnson et al., 2007). The effects of copper may have gone undetected in our investigation due to biological variation or insufficient levels of hair cell damage, as they cannot be explained by alterations in baseline activity of larvae. Nevertheless, rheotaxis tests proved more sensitive than the startle assay on comparison (Table 5.5).

### 5.11.2 Seeker response

The seeker response assay was the final test used to assess lateral line function following ototoxin treatment. Seeker response testing aimed to answer two questions: are larvae responsive to water flow disturbances and do they retain neuromuscular function after compound exposure? A previous study of predator evasion showed that neomycin treatment causes a decreased

responsiveness to directional water flow, reducing the escape manoeuvre (McHenry et al., 2009). This published result led to the hypothesis that treatment with ototoxins would cause a decrease in SR score. In the current study, there was an observable decrease in SR score for all of the positive compounds tested. This reduction in score was significant even at the $IC_{50}$ level. It was also hypothesised that negative compounds and solvent control treatments would have no effect on SR score. This was true for all negative compounds, except melphalan, which gave an unexpected minor decrease in SR score. This result could not be attributed to a sedative effect, as melphalan treatment did not alter the activity profile of the larvae. Reduction in SR score is the third functional consequence of hair cell damage to the lateral line to be detected here.

## 5.12 Sedation and seizure induction do not account for alterations in functional responses to exogenous stimuli

To eliminate the possibility that the decrease in startle response caused by ototoxins was due to sedative effects, the activity profiles of the larvae from baseline recordings were analysed. With the exception of neomycin, none of the test substances had a significant sedative-type effect on the overall movement of larvae. When neomycin treatment was compared to immersion in the anaesthetic MS222, it appeared that the decreased activity caused by neomycin exposure may have been due to slight sedative effects. Interestingly, the decrease in baseline activity of neomycin-treated larvae could not solely be attributed to sedative effects, as neomycin treatment had a stronger effect on rheotaxis and seeker response than light sedation. These findings indicate that perhaps a more subtle, compound-specific effect is occurring with neomycin treatment that cannot be explained by sedation alone.

## 5.13 Concluding remarks

The startle, rheotaxis and seeker response assays were used to assess the functional consequences of exposure to ototoxins and negative control compounds. The data shown demonstrate that larval zebrafish can experience deficits in detecting auditory and vibratory stimuli, and in orientation to current flow, following hair cell damage induced by a range of ototoxins. In combination, these assays provide a wealth of information on the ototoxic potential of

compounds. Taken together, they support the theory that larval zebrafish responses mimic the functional hearing and vestibular effects seen in humans and non-human mammals following exposure to certain ototoxins.

# Chapter 6 Discussion and future directions

## 6.1 Discussion

### 6.1.1 Project aims

The overall goal of this study was to assess the true value of the larval zebrafish as an in vivo model of drug-induced hearing and balance impairment. To test this, the main experimental aims were:

(i) To demonstrate the damage induced to hair cells by a range of known human ototoxins (using vital dye uptake and expression assays).
(ii) To develop a set of novel assays to investigate the functional consequences of this hair cell damage.
(iii) To investigate the underlying mechanisms of ototoxicity in one or more of the selected test compounds and to assess the translational capability of the zebrafish as a model, by comparing the results to what is known from mammalian studies.

Aims (i) and (ii) were fulfilled, with assays developed and successfully utilised to show both histological and functional damage to hair cells following exposure to a range of ototoxins. The completion of this body of work resulted in a publication (Buck et al., 2012). This chapter summarises and discusses these results. In addition, some data were generated in support of understanding the mechanisms of cisplatin-induced ototoxicity in the zebrafish (aim iii). The results and progress towards this aim are described in this Chapter and in Appendix 3.

### 6.1.2 Hair cell toxicity in the zebrafish PLL

In Chapter 3 of this thesis, a combination of approaches was utilised in order to assess the damage induced to hair cells following exposure to human ototoxins.

Vital dye and TUNEL staining was performed in order to examine the damage induced to the hair cells by a range of human ototoxins. Experiments using the vital dyes DASPEI and FM1-43FX demonstrated that all except one of the human ototoxins tested (furosemide) could damage hair cells in the zebrafish lateral line in a concentration-dependent manner. A reduction in GFP labelling was also observed in the *Tg(pou4f3::mGFP)s356t* line in response to ototoxin

treatment. More specifically, TUNEL staining was observed in larvae treated with neomycin; this was indicative of apoptotic hair cell death.

The ototoxins tested were selected from a range of therapeutic classes: the aminoglycoside antibiotics, platinum based chemotherapeutics, loop diuretics, salicylates and heavy metals (See Chapter 2, Table 2.1). The DASPEI assay had a predictivity rate of 85% for these compounds (6 out of 7 detected as ototoxins). This highlights the relative versatility of the zebrafish lateral line in assessing different classes of ototoxin. A predictivity power of >85% is classed as 'excellent' by the European Centre for the Validation of Alternative Methods (Genschow et al., 2002 as reviewed by; Redfern et al., 2008). Seventy-five percent (3 out of 4) of negative control compounds were identified as non-toxic to hair cells.

Overall, the data obtained in this section of the study correlate well with the results from other laboratories working in zebrafish (Chiu et al., 2008; Coffin et al., 2010; Harris et al., 2003; Hernandez et al., 2006; Ou et al., 2007; Ou et al., 2010; Owens et al., 2007; Owens et al., 2009; Ton and Parng, 2005; Van Trump et al., 2010). In particular, the slow onset of cisplatin ototoxicity, the threshold effects of copper sulphate toxicity in hair cells and the apoptotic-like changes observed with neomycin-induced hair cell damage have also been observed by others (Dr. Clemens Grabher personal communication; Ou et al., 2007; Owens et al., 2007).

Aspirin is a known human ototoxin that reportedly causes reversible hearing loss and tinnitus. Encouragingly, the data obtained in this study using the mitochondrial vital dye DASPEI has shown that aspirin can cause hair cell damage in the zebrafish lateral line. This has not previously been demonstrated in zebrafish.

In order to contextualise the results obtained in this investigation, the effective concentrations to elicit hair cell damage according to the DASPEI assay were compared to those required to damage hair cells in higher vertebrates. In general, the concentrations required to elicit hair cell damage in the posterior lateral line correlated best with published data from explanted tissue culture in mice.

For example, the concentration to completely abolish DASPEI staining in the lateral line was 100 µM compared with recorded damage to the basal coil of the mouse cochlea at 200-250 µM after the same exposure time (Kotecha and Richardson, 1994; Richardson and Russell, 1991). This damage was seen most clearly at the apical surface of the hair cells in cochlear cultures, with a higher concentration of neomycin required to elicit damage at the apical coil structures (Richardson and Russell, 1991). In a separate study, the concentration reported to cause severe damage to hair cells in Organ of Corti explants from rats was 100 µM (Mazurek et al., 2012), however this was after a much longer compound exposure (48 hours). In organ of Corti explants taken from human embryonic tissue, the concentration of neomycin required to elicit such hair cell damage is higher (1 mM; Mu et al., 1996). The more variable results from explant experiments are still within a 10-fold change from the concentrations used in zebrafish in this study.

The ototoxin gentamicin can severely damage hair cells from mouse cochlear explants at a concentration of 250 µM after a one hour exposure (Kotecha and Richardson, 1994). At this concentration in the pLL of the zebrafish, there is also a large and significant decrease in hair cell staining in the lateral line. Additionally, gentamicin is toxic to HEI-OC1 cells at a concentration of 50 µM after prolonged exposure (24 hours; Kalinec et al., 2005). Although not tested this lower concentration would most likely have a strong damaging effect upon hair cells of the lateral line after chronic immersion, as this study estimates that the $IC_{50}$ for gentamicin exposure after a single one hour treatment is 24.75 µM.

Experiments using cisplatin have shown that hair cell death can be elicited by chronic treatment in both explants and HEI-OC1 cells. The concentration required to damage or destroy half of the hair cells in explants from rat cochleae was given as 33 µM following a 48 hour exposure (Malgrange et al., 1998). In the HEI-OC1 line, an 8 µg/mL dose (equivalent to ~27 µM) over 24 hours was sufficient to kill all of the organ of Corti-derived cells (Lee et al., 2011). In the current study, acute 2 hour exposures of batch 029K1426 of cisplatin were severely toxic to hair cells at a concentration of 70-100 µM. It is entirely feasible

therefore that a longer low-dose treatment could cause the same level of hair cell damage in the zebrafish.

The comparison between compound-induced damage in the zebrafish larva and in vivo results taken from higher vertebrates is much harder to make, as dosing regimens are often repeated over longer periods of time (i.e. one week) and ABR measurements and histological samples are taken after an additional period following cessation of dosing (i.e. 30 days post treatment). A further barrier to direct comparison is that the compound pharmacokinetics and the penetration of the compound into the hair cells are much more difficult to assess, therefore the effective concentration to destroy the hair cells is much harder to estimate. This is not the case in the zebrafish, as the hair cells are directly exposed to the solution containing the compound and therefore a good estimate of the effective concentration can be made. It is however known that repeated dosing with neomycin, gentamicin and cisplatin (for example) in higher vertebrates, such as guinea pigs, can cause significant shifts in ABR and compound action potential (CAP) thresholds and significant disruption to hair cell structure.

Taken together, the results of histological assays for ototoxicity in the zebrafish lateral line appear to be robust and highly reproducible between laboratories, and are comparable to other studies, including those using the explanted tissues of higher vertebrates.

### 6.1.3 Hair cell toxicity in the zebrafish ear

In addition to investigations in the lateral line, it was also desirable to assess the effects of ototoxins on the zebrafish ear (Chapter 3). The aims were to discern if ototoxins could also damage hair cells of the maculae and cristae and to determine whether the damage induced was specific to certain sensory patches of the ear, thus mimicking the damage in humans and other vertebrates. Previously, an otic injection technique had been used to label hair cells (Seiler and Nicolson, 1999). A similar technique was optimised in this study in order to inject ototoxins into transgenic *i193* larvae. As in the lateral line, the ototoxins tested damaged hair cells within the ear. The damage induced appeared to act preferentially on the posterior macula, although it was uncertain to what extent

ear development affected the specificity of the ototoxins to damage the saccule. In zebrafish the posterior (saccular) macula is proposed to have mainly auditory function. It was not possible to conclude whether the damage to the saccule in zebrafish would be the equivalent of damage to the auditory organ of higher vertebrates (the cochlea) or to the tissue of the saccule in higher vertebrates, as no functional readout could be taken.

Taken together, these data demonstrated that the hair cells of both the zebrafish lateral line and inner ear can be damaged by exposure to known human ototoxins, mimicking the pathological findings from numerous studies in higher vertebrates.

### 6.1.4 The functional effects of ototoxins on the lateral line

Prior to this study, very few findings had been published that addressed the functional consequences of hair cell damage in the larval zebrafish subsequent to ototoxin exposure. Prior to the current study, only two publications showed results from functional testing in the larval zebrafish following ototoxin exposure. One study (Johnson et al., 2007), assessed the rheotactic behaviour of larval zebrafish following copper exposure as part of a wider study to assess the effects of this compound on the morphological and functional development of embryos. A second study illustrated the effects of neomycin on the tactile escape response by monitoring escape caused by accelerated water flow (McHenry et al., 2009). Neither of these studies set out to specifically investigate the ototoxic effects of a variety of compounds or a range of functional readouts.

It is well known that the lateral line can mediate reflex behaviours such as the escape response and rheotaxis in adult and larval fish of other species. A set of novel assays were developed that could be used in combination to assess lateral line function in larvae following ototoxin exposure. An assay was developed and optimised to examine larval auditory evoked startle responses following compound exposure; this assay was based partly on the work of David Zeddies and Richard Fay and on a study by Pascal Bang (Bang et al., 2002; Zeddies and Fay, 2005). In addition, methods to assess larval rheotaxis and underwater motion detection were developed.

Data from the optimisation of the startle assay revealed a number of interesting outcomes. Factors such as plate type, raising density and inter-stimulus interval were all able to alter the control compliance (baseline response) of animals to a pure tone stimulus (For a detailed discussion, refer to Chapter 4). By optimising for these factors, it was possible to increase the quality of the startle assay.

Startle, rheotaxis and seeker response were assessed consecutively in a final functional testing paradigm (Chapter 5 and Appendix 2). These assessments produced a large amount of valuable data for each of the test compounds (AER value, baseline activity value, compound toxicity, rheotaxis score, SR score), indicating their potential ototoxicity. The predictivity of the data was good for each of the assays; 67% for the startle assay ('sufficient' according to EVCAM), 83% for the rheotaxis test, and 100% for the seeker response test (both 'excellent' according to EVCAM). It was decided that due to its lower predictive value, the startle assay should not be used alone to identify ototoxins, but was useful in conjunction with the additional assays. By analysing these results together, a better overall indication of the specific hair cell toxicity of the compounds could be obtained. If the functional assays were to be developed further into a hierarchical screening method, then the recommendation would be to use the three functional assays in combination initially, as a 'first-tier' screen. Secondary testing using the DASPEI assay could be then used to confirm any 'hits' identified.

An attempt was made to assess the effects of ototoxic compounds individually at isolated frequencies. It was hypothesised that damage to the lateral line would affect the lower frequencies (up to ~200 Hz) more than the higher frequencies. Unfortunately, no pattern of damage at specific frequencies could be observed. A more positive outcome of supplementary tests using neomycin was the ability of the zebrafish AER testing to mimic ABR threshold shift testing in higher vertebrates; it was possible to rescue the AER by increasing the stimulus volume in increments. Importantly, the startle assay gives an indication of the state of a functional response that is highly specific to hair cells and is a simple alternative assay to mammalian ABR testing, which is far more time-consuming and costly.

Since the publication of this body of work (Buck et al., 2012), two new studies have been published, both concerned with the use of larval zebrafish to assess rheotaxis (Olszewski et al., 2012; Suli et al., 2012). These studies both showed that neomycin treatment could significantly attenuate rheotactic behaviour at concentrations between 50 and 400 µM. The data presented in this study showed that a significant reduction in larval rheotaxis could be seen at concentrations as low as 14 µM. These publications lend further support to the data presented in this thesis concerning rheotaxis, suggesting that the results of this assay in particular are reliable. It is also encouraging to see that there has been a shift of focus within the field to a more functional approach to test the ototoxic effects of compounds.

### 6.1.5 Comparative sensitivities of the ototoxicity assays

Assays were compared directly for each of the ototoxins tested. The most sensitive assay was decided first by lowest detectable concentration effect (LOEC) and then by significance level (α) at that concentration. When all of the assays were compared, the seeker response and DASPEI assays emerged as the most sensitive. These assays can detect alterations in hair cell morphology and lateral line function at lower concentrations and with more accuracy than the startle and rheotaxis assays. Specifically, the DASPEI assay is the best assay to detect ototoxicity caused by neomycin, streptomycin and aspirin, whereas the SR assay is the best assay to detect damage caused by gentamicin, cisplatin and copper sulphate (see Chapter 5, Table 5.5). This highlights the importance of using more than one assay to assess a single outcome.

### 6.1.6 Further validation of the ototoxicity assays

The next logical step for this work from a safety screening perspective would be to perform a blinded validation screen using ~25 compounds, like those performed for other safety endpoints such as seizure liability, gut motility and visual function (Berghmans et al., 2008; Richards et al., 2008; Winter et al., 2008). If results from this validation set showed good sensitivity, it would then be possible to carry out further in-house validation on a large scale. The eventual application of this work would be for pharmaceutical companies to incorporate the ototoxicity assay into a hierarchy of safety screens at the ‘Lead

Identification' and 'Lead Optimisation' stages of compound development. These are early stages of drug discovery where chemical modification to the molecular structure is still possible, to reduce safety liabilities (Stevens and Baker, 2009). Any potential ototoxins could be flagged up at the early stages for further evaluation in a lower throughput study using a higher vertebrate. The use of these functional tests for an ototoxicity screen may however require some more investigation into the automation of certain processes. For example, automatic recording by the camera upon stimulation could be developed. The future development of the assays described here is outside of the remit of this project.

### 6.1.7 Caveats

There are some caveats to the use of zebrafish in assessing ototoxicity.

Firstly, any assessment of a new chemical entity only investigates the effects on the hair cells, and therefore compounds that affect any other part of the ear may go undetected. For example, the loop diuretic furosemide was not detected as an ototoxin in this and another investigation in zebrafish (see Chapter 3; Chiu et al., 2008). This is likely to be because furosemide is supposed to act to alter endolymphatic fluid composition and not to damage the hair cells of the lateral line. Additionally, it is unlikely that the effects of this compound would be seen by fluid volume changes in the otic vesicle (a readout of fluid misregulation in the ear) because the channel that furosemide functions through (NKCC1) probably lacks the critical residues for this role in zebrafish (Abbas and Whitfield, 2009; Delpire et al., 1999).

When assessing the ototoxins it is also important to note that the hair cells of the lateral line and ear in zebrafish are also more similar to vestibular than cochlear hair cell types in mammals (Owens et al., 2007). Any results acquired are therefore most likely to reflect changes to the vestibular epithelium in humans and not the cochlea.

As shown in this and other studies, the duration of exposure to ototoxins can have a significant effect on the results seen. For example, acute exposure of larvae to cisplatin for 30 minutes would not result in hair cell toxicity, as the effects of the compound on the hair cells can only be seen after 2 hours. It is therefore important to bear in mind that not all potential ototoxins will be

detected within a short exposure time, and to design experiments accordingly. This would be particularly relevant if a validation screen were performed where compounds were tested only for a single exposure time.

Finally, it is important to remember that all of the experiments described have been carried out as acute exposures. In reality however, the damage caused in humans is often a consequence of chronic and cumulative exposures.

The introduction to this thesis outlined that “any proposed model (of ototoxicity) must confer the advantages of the mammalian, avian and in vitro systems and still be cost-effective, reliable and show good predictivity”. The concordance between the data for explants and the data for zebrafish from the DASPEI assay provides support for the notion that the zebrafish can confer the advantages of an in vitro system. The tissues under investigation are easily accessible (less time consuming) and any compound-induced changes are obvious, allowing for good throughput. The DASPEI assay in itself is robust and predictive. Importantly, the zebrafish lack some of the disadvantages of using in vitro systems. Notably, the zebrafish hair cells are contained within neuromasts along with supporting cells, offering a more complex cellular environment in which to study ototoxicity than simpler cell line assays. Also the hair cells of the lateral line and ear are not vulnerable to the genotypic and phenotypic changes that cell lines can undergo as a consequence of multiple passages (e.g. HEI-OC1 cell line; Cederroth, 2012). The zebrafish also confer some of the advantages of mammalian systems. As in higher vertebrates, the functional consequences of hair cell toxicity can be readily observed, in a reproducible manner. Additionally, genetic tools such as transgenic animals can be harnessed to better understand the effects of ototoxicity. The zebrafish is an elegant and simple model to identify hair cell toxins with acceptable levels of predictivity and with higher throughput and lower costs than any mammalian assay. It is unlikely that the zebrafish will ever act alone as a *replacement* for in vivo mammalian investigations into ototoxicity. However, the zebrafish assays are certainly a useful and informative tool with numerous advantages that could be used in combination with assays in higher vertebrates and cell lines. If placed into a hierarchy of early pre-clinical screening, they could enable potential ototoxic effects of new chemical entities to be flagged up for further

testing in higher organisms. This is a vast improvement on the current situation, where ototoxicity is not addressed at all in the pharmaceutical industry.

## 6.2 Future Directions

In order to fully determine the potential of the zebrafish as a model of ototoxicity, it would be desirable to assess the similarities and differences in mechanisms of ototoxicity between zebrafish and non-human mammals in a separate follow-on project. Ideally, any ototoxic compound under investigation would have been studied in detail previously in the more traditional animal models such as mice, rats and guinea pigs.

To date, work by other zebrafish laboratories has focussed predominantly on elucidating the underlying mechanisms of aminoglycoside-induced hair cell death (as briefly summarised below). The results from this thesis have highlighted a number of potential avenues for future investigation that are not concerned with the aminoglycoside antibiotics.

Data from Chapter 3 have shown for the first time that the human ototoxin aspirin can damage hair cells in the zebrafish lateral line. It would be of value to further dissect the mechanisms of this damage and draw some comparisons to what is known from the in vivo literature from non-human mammals. The areas of aspirin-induced hair cell death to investigate are detailed in Section 6.3.1.

Another area that is poorly investigated in the zebrafish concerns the underlying mechanisms of cisplatin-induced hair cell death. Initial investigations into the underlying mechanisms of cisplatin-induced hair cell damage were begun towards the end of this thesis, providing some exciting preliminary findings (see Appendix 3). These preliminary data could readily form the basis of a new grant proposal. The continuation of this work is a priority in fully validating the zebrafish as a model of ototoxicity. For this reason, the potential of the zebrafish in assessing cisplatin-induced toxicity is discussed below (Sections 6.2.1.3 and 6.3.2).

### 6.2.1 Mechanisms of ototoxicity: species comparison.

6.2.1.1 Aminoglycoside antibiotics:

Knowledge from in vitro and in vivo studies in other models

As discussed in detail earlier (see Chapter 1) the best explanations for the mechanisms of damage inside auditory cells are the stimulation of reactive

oxygen (and possibly reactive nitrogen) species production by the formation of heavy metal-aminoglycoside complexes and polyphosphoinositide lipid-aminoglycoside binding, leading to generation of lipid peroxides. Based on the high volume of evidence, caspase-dependent apoptosis, involving the mitochondria, is likely to be a primary cell death event in aminoglycoside-induced ototoxicity. Some necrotic events have also been less frequently reported. Many otoprotective strategies have been employed against aminoglycoside-induced hair cell loss. These protection studies have enabled researchers to uncover the pathways that are most likely to mediate hair cell damage. For example, inhibition of caspases and JNK signalling can protect hair cells from death (Matsui et al., 2003; Okuda et al., 2005; Pirvola et al., 2000; Ylikoski et al., 2002).

Current knowledge in zebrafish

Aminoglycoside-induced ototoxicity is a popular research area within the zebrafish field. Not only have the cellular consequences of aminoglycoside-induced damage been described, but also major laboratories have focussed their efforts on the discovery of compounds that can protect hair cells from aminoglycosides. As with other model systems, the discovery of protectants in zebrafish provides tantalising clues as to the possible mechanisms underlying the damage.

The ototoxicity of various aminoglycosides (neomycin, gentamicin, streptomycin, tobramycin, kanamycin, amikacin) is well reported in the zebrafish (Harris et al., 2003; Owens et al., 2009; Seiler and Nicolson, 1999; Williams and Holder, 2000). Damage to the hair cells by aminoglycosides has been evaluated using electron microscopy techniques. Studies of this damage have shown loss of hair cell bodies and stereocilia bundles, some extrusion of hair cells from the apical membrane, mitochondrial alterations, apoptotic and necrotic debris (Harris et al., 2003; Murakami et al., 2003; Owens et al., 2007). This is in agreement with histological evidence from other model systems (e.g. De Groot et al., 1991; Dehne et al., 2002; Richardson and Russell, 1991; Ylikoski et al., 2002).

The mechanism of uptake of the aminoglycosides in zebrafish is proposed to be via mechanotransduction channels at the apical membrane of hair cells, mimicking in part what has been suggested in other model systems (Alharazneh et al., 2011; Gale et al., 2001; Kossl et al., 1990). This has been investigated using fluorescently conjugated aminoglycosides and chemical inhibitors such as amiloride (Coffin et al., 2009; Ou et al., 2009; Seiler and Nicolson, 1999; Wang and Steyger, 2009). The mechanotransductive uptake described is also known to be affected by extracellular divalent cations such as calcium, with altered levels of calcium conferring protection or promoting aminoglycoside-induced cell death (Coffin et al., 2009; Wang and Steyger, 2009). Like in mammals, the apoptotic death observed is thought to involve early changes to the mitochondria. For example, it has been shown using vital dye labelling that the mitochondrial membrane potential of the hair cells is reduced in response to acute neomycin exposure (Owens et al., 2007). Further investigations into this class of antibiotics suggests that there may be two phases of compound-induced cell death, an early and late phase, and this suggests that some members of the aminoglycosides may undergo hair cell death via divergent mechanisms (Owens et al., 2009).

Many screens have been carried out in the zebrafish in order to discover protectants against aminoglycoside-induced ototoxicity (Ou et al., 2009; Ou et al., 2012; Owens et al., 2008; Vlasits et al., 2012). These protection assays can provide clues as to which signalling pathways may mediate hair cell loss. For example, two benzothiophene carboxamide derivatives (PROTO1 and PROTO2) most likely act intracellularly to attenuate hair cell toxicity (the mechanism by which they act is still under investigation) (Owens et al., 2008). The *Sentinel* mutant confers protection against hair cell death caused by the aminoglycosides but not cisplatin, whereas some drugs (such as benzamil) protect against both cisplatin and aminoglycoside-induced hair cell loss (Owens et al., 2008; Vlasits et al., 2012). This suggests that there are both shared and divergent pathways of hair cell death involved between the two drug classes. Most recently, quinoline-derived compounds have been shown to protect hair cells from aminoglycoside-induced death. These quinoline-derivates appear to

exert their protective effects by inhibiting the uptake of the aminoglycosides (Ou et al., 2012).

#### 6.2.1.2 Aspirin

As mentioned, the ototoxic effects of aspirin have not previously been reported in the zebrafish. It will therefore be interesting to investigate this hair cell damage in more detail.

The temporary hair cell damage induced in humans and mammals during aspirin treatment has been suggested to involve the motor protein prestin. Prestin is the motor protein of outer hair cells that functions in cochlear amplification. It is a direct voltage-to-force converter, using anions as voltage sensors (Dallos and Fakler, 2002). Studies using the patch clamp technique in isolated outer hair cells from the rat and guinea-pig have shown that aspirin acts as a competitive antagonist at the anion-binding site of prestin; the hypothesis is that aspirin interferes with electromotility of the hair cells and thus disrupts cochlear amplification (Homma and Dallos, 2011; Kakehata and Santos-Sacchi, 1996; Oliver et al., 2001; Tunstall et al., 1995). Prestin is expressed in zebrafish ears at both larval and adult stages and acts as a voltage sensor (Albert et al., 2007; Weber et al., 2003). It would be interesting to assess the role of prestin in aspirin-induced ototoxicity in the zebrafish (see Section 6.3.1).

The transient tinnitus provoked by aspirin is thought to be caused through the overactivation of NMDA receptors (Guitton et al., 2003; Peng et al., 2003; Puel and Guitton, 2007). Behavioural assays have been developed in rats to observe tinnitus induced by compounds including aspirin (Jastreboff et al., 1988; Ruttiger et al., 2003). In general, these assays work by exposing the animal to prolonged white noise and conditioning them to perform a set task (such as feeding) during this white noise. Gaps or sinusoidal tones are placed within the noise, which can cause the animals to react by altering their behaviour (i.e. stop pressing a lever for food, as in Bauer et al., 1999). Tinnitus is inferred when the animals fail to alter their behaviour in response to the stimulus (the silence or tone). In rats, it has been shown that tinnitus-like behaviour can be reduced by NMDA receptor antagonists such as MK-801 and gacyclidine (Guitton et al., 2003). It may be useful to develop a behavioural assay for tinnitus in the

zebrafish model and compare results to what is known from non-human mammalian data.

#### 6.2.1.3 Cisplatin

In recent years, a large volume of research has been undertaken in both cell lines and the more traditional animal models in order to understand the underlying mechanisms of cisplatin-induced hair cell toxicity better. Studies have focussed not only on the potential mechanisms of uptake of the compound itself, but also on the complex downstream signalling pathways which culminate in the death of the hair cells themselves. What follows is a review of the literature thus far (for a summary, see Figure 6.1).

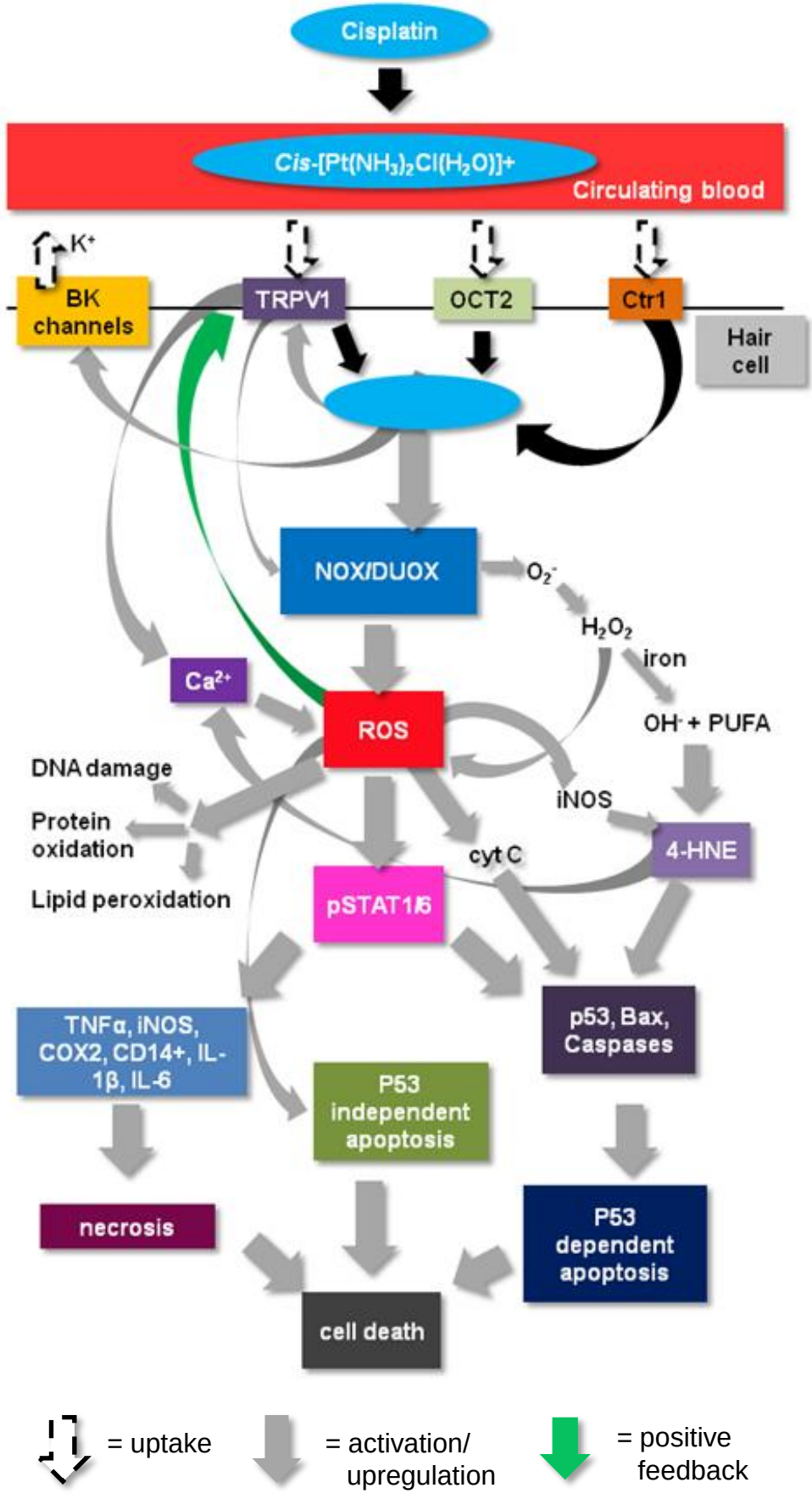



**Figure 6.1: Proposed mechanisms of cisplatin-induced hair cell death compiled from mammalian and cell culture data.**

Uptake of cisplatin into hair cells

Two main mechanisms of cisplatin uptake into cells have been proposed in recent years. The first is that cisplatin enters the hair cells via Organic Cation Transporter 2 (OCT2). The second channel proposed as the key mediator of cisplatin uptake is Copper Transporter 1 (Ctr1).

There is evidence that OCT2 is the major uptake channel for cisplatin. OCT2 (SLC22A2) is an electrogenic transporter of cations. It was first isolated from homology screening in the rat kidney (Okuda et al., 1996). The channel possesses twelve α-helical transmembrane domains (TMDs), with a large extracellular loop between TMDs one and two and a large intracellular domain between TMDs six and seven (Ciarimboli, 2011). Cisplatin is known to interact with human OCT2 and use it as a channel for entry into cells (Burger et al., 2010; Ciarimboli et al., 2005; Yonezawa and Inui, 2011). In humans, single nucleotide polymorphisms in the *SLC22A2* gene are associated with reduced cisplatin ototoxicity and nephrotoxicity (as reviewed by Deavall et al., 2012). In mice, the OCT2 protein has been shown to be expressed specifically in the apical pole of outer hair cells and the entirety of the inner hair cells, with some additional expression in the stria vascularis (Ciarimboli et al., 2010; More et al., 2010). The most convincing evidence for the role of OCT2 in cisplatin uptake comes from protection experiments. Cimetidine, a competitive substrate for OCT2, is protective to hair cells both in vitro and in vivo and can confer complete protection against the ototoxic effects of the compound (Ciarimboli et al., 2005; Ciarimboli et al., 2010). Knockout mice lacking the *OCT2* gene were significantly protected from ototoxicity, as shown by ABR measurements (Ciarimboli et al., 2010). OCT2 is the ideal candidate for otoprotective therapy, as it does not appear to be involved in the uptake of cisplatin into tumours; thus knocking down or chemically inhibiting OCT2 should not interfere with the anticancer effects of the compound (Ciarimboli et al., 2010).

Interestingly, there is also some evidence that Ctr1 could be a channel for cisplatin uptake into cells. Human Ctr1 has three transmembrane domains, an N-terminal extracellular domain, a large intracellular loop, and a short intracellular C-terminal tail (Abada and Howell, 2010). It sits in the membrane as

a trimer; this trimeric formation creates a pore through the cell membrane (De Feo et al., 2009). Ctr1 protein has been shown to be expressed in mouse cochlear lysate, HEI-OC1 cells and in the mouse cochlea. Specifically, expression can be seen in the organ of Corti. The expression of Ctr1 protein in Human Embryonic Kidney (HEK) and HEI-OC1 cells was shown by the MTT assay to enhance cytotoxicity. Moreover, co-administration of cisplatin and copper sulphate by intratympanic injection had a partial yet significant protective effect on auditory function (More et al., 2010).

In addition, investigations have shown that cisplatin and reactive oxygen species (produced downstream by cisplatin) may increase the activation of the Transient Receptor Potential channel, TRPV1 (Mukherjea et al., 2008). The overactivation of TRPV1 could act as a positive feedback loop, further activating NOX enzymes (e.g. NOX-3) and increasing ROS levels and damage via calcium influx into the cells (Mukherjea et al., 2011). Importantly, TRPV1 may also act as a permeant channel for cisplatin, further augmenting the process (reviewed by Rybak et al., 2009). The protective effect of the TRPV1 inhibitor capsazepine gives further support to this potential mechanism (Mukherjea et al., 2008).

Early downstream targets of cisplatin

Cisplatin is known not only to activate TRPV1 channels upon entry into the cell but has also been shown to activate big conductance potassium channels (BK channels). The activation of these channels in the lateral wall of the inner ear leads to increased potassium efflux and disrupts the delicate electrochemical gradient in place, resulting in cell death (Liang et al., 2005).

Of particular interest in recent years, the NOX family of NADPH oxidase enzymes are thought to play a key role in cisplatin ototoxicity. The NOX family transfer electrons across biological membranes. In other cell types they function to generate reactive oxygen species (Bedard and Krause, 2007). One of the NOX enzymes, NOX-3 has been shown specifically to be expressed in the organ of Corti and vestibular system of mammals (Banfi et al., 2004; Paffenholz et al., 2004). The NADPH oxidases NOX-1 and NOX-4 are expressed in HEI-OC1 cells (Kim et al., 2011). The NOX enzymes are upregulated in hair cells by

cisplatin and additionally via activated TRPV1 channels (Kaur et al., 2011; Mukherjea et al., 2006; Mukherjea et al., 2011). When the levels of NOX enzymes in the cell increase, reactive oxygen species are generated; this causes widespread cellular damage and death. Additionally, superoxides produced by NOX enzymes go on to generate highly toxic 4-HNE downstream, leading to increased cell death. Small interference RNA (siRNA) to knock down NOX-1 and -4 has been shown to protect HEI-OC1 cells from apoptosis (Kim et al., 2011). Likewise, siRNA knock down of NOX-3 in the rat is protective, decreasing ROS and phosphorylated STAT levels downstream (Kaur et al., 2011). Chemical inhibition of NADPH oxidases has been shown to confer significant protection against cisplatin-induced hair cell damage (Choi et al., 2011; Kaur et al., 2011; Kim et al., 2011). For example, DPI, a pan-NOX inhibitor protects both UB/OC-1 and HEI-OC1 cells from death induced by cisplatin (Kaur et al., 2011; Kim et al., 2011).

As mentioned above, cisplatin generates high levels of reactive oxygen species that act to overwhelm the cell and deplete antioxidant levels (Clerici et al., 1996; Kopke et al., 1997). Many thiol-based antioxidants have been shown to confer protection to hair cells by acting against these reactive oxygen species and "mopping up" the cisplatin in the cell (reviewed in Rybak et al., 2009). For example, sodium thiosulphate protects against functional changes in hearing and against hair cell loss in the cochlea of guinea pigs (Berglin et al., 2011; Wang et al., 2003a; Wimmer et al., 2004). The antioxidants D-Methionine (D-Met) and N-acetyl-L-cysteine (L-NAC) offer otoprotection in cell culture and explants of the stria vascularis and organ of Corti (Campbell et al., 1996; Campbell et al., 1999; Feghali et al., 2001). Encouragingly, a recent study in humans has also found that transtympanic administration of L-NAC is protective against changes in auditory function (Riga et al., 2011).

Evidence suggests that phosphorylation of the transcription factors STAT1 and STAT6 occurs as a consequence of high levels of reactive oxygen species in both the auditory and vestibular cells following cisplatin exposure. In cultured mouse utricles phosphorylated STAT1 (pSTAT1) peaks 4 hours after cisplatin exposure (Schmitt et al., 2009). In UB/OC-1 cells, pSTAT1 activation can be seen within 45 minutes. In rat cochlea pSTAT1 is upregulated following a 72

hour cisplatin administration (Kaur et al., 2011). pSTAT6 is also upregulated in HEI-OC1 cells following cisplatin treatment (Kim et al., 2011). In all cases where STAT phosphorylation was increased, preventing this phosphorylation using siRNA knock down or chemical inhibition had a significant protective effect (Kaur et al., 2011; Kim et al., 2011; Schmitt et al., 2009). Moreover, STAT1 and STAT6 deficient mice are resistant to the ototoxic effects of cisplatin (Kim et al., 2011; Schmitt et al., 2009). Translocation of phosphorylated STATs to the nucleus triggers downstream cell death events involving both apoptotic and inflammatory pathways.

Additional downstream effects of ROS are increased production of the nitric oxide synthase “iNOS” (which can in turn lead to increases in 4-NHE), the release of cytochrome C from the mitochondria (triggering downstream apoptotic events), protein oxidation, lipid peroxidation and DNA damage.

Both apoptotic and necrotic like cell death pathways have been implicated in cisplatin-induced ototoxicity. These effects have been observed in auditory- and vestibular-type cells. Phosphorylated STATs have been shown to activate both pathways, inducing not only increased caspase levels but also increases in pro-inflammatory cytokines such as TNFα and IL-1β and IL-6 and inflammatory CD14-positive cells (Kaur et al., 2011; Kim et al., 2011; Mukherjea et al., 2011; So et al., 2007; So et al., 2008). Inhibition of caspases, p53, iNOS and inflammatory cytokines protects cells from ototoxic insult induced by cisplatin, further providing evidence that cell death occurs by both routes (Cooper et al., 2006; Kaur et al., 2011; Kelly et al., 2003; Kim et al., 2008; Kim et al., 2011; Lee et al., 2010; So et al., 2007; So et al., 2008; Wang et al., 2004; Zhang et al., 2003).

Current knowledge of cisplatin ototoxicity in the zebrafish

To date, the literature concerning the ototoxic effects of cisplatin in the zebrafish is sparse.

A study by Ton and Parng published in 2005 was the first to show that chronic cisplatin exposure (24 hours) could induce hair cell damage in the zebrafish. Subsequently, a more comprehensive study was performed in order to assess

cisplatin toxicity in more detail (Ou et al., 2007). This study showed that acute cisplatin exposure (4 hours) induced repeatable and predictable damage to lateral line hair cells. Cisplatin damage continued even after removal of the drug and was independent of the initial exposure concentration.

The time course of cisplatin-induced ototoxicity is known to be slower than for neomycin in zebrafish (Ou et al., 2007; Owens et al., 2009). Ou and colleagues proposed that the different time course for damage could be explained by the distinct mechanisms of uptake, cellular damage and cellular clearance by the two compound classes. Evidence from mammalian studies suggests that neomycin and cisplatin act in part through different damage pathways; for example Jun kinase inhibition blocks neomycin but not cisplatin-induced hair cell death in guinea pigs (Wang et al., 2004). Although not investigated in the paper by Ou, a later paper from the same laboratory showed that otoprotectants (a mutation in the *sentinel* gene and the compound PROTO-1) that could significantly protect from neomycin induced hair cell death could not confer protection to cisplatin-treated animals (Owens et al., 2008). This suggested that there were in fact some distinct mechanisms of early damage events or compound uptake between neomycin and cisplatin.

Conversely, there is evidence of *similarities* in the late damage mechanisms between cisplatin and neomycin in the literature taken from mammalian and cell line research. Both compounds are known to promote formation of reactive oxygen species and caspase inhibitors are protective against their ototoxic effects, suggesting that both cascades end in apoptotic death. There is evidence in the zebrafish that cell damage mechanisms must be at least in part overlapping; the compounds benzamil and paroxetine can protect against both neomycin and cisplatin induced hair cell loss (Vlasits et al., 2012).

Interestingly, a number of antioxidant compounds have been shown to confer protection to the hair cells of larval zebrafish during cisplatin exposure. Some of these compounds also have a protective action in higher vertebrates. Co-treatment with the antioxidants glutathione (GSH), allopurinol (ALO), *N*-acetyl L-cysteine (L-NAC), 2-oxothiazolidine-4-carboxylate (OTC) and D-methionine (D-MET) prevented decreases in hair cell staining with DASPEI, indicative of

protection (Ton and Parng, 2005). Additionally, the polyphenolic compound epicatechin was able to protect hair cells of the zebrafish lateral line from cisplatin ototoxicity; this was shown using the vital dye YO-PRO1 and by electron microscopy (Kim et al., 2008). These results imply that cisplatin also induces the formation of reactive oxygen species in zebrafish.

## 6.3 Opportunities for further work/experiments

### 6.3.1 Aspirin

As mentioned above, the finding that the human ototoxin aspirin is also toxic to hair cells of the zebrafish lateral line is novel. A number of future follow-on experiments could be performed to build on this initial discovery.

#### 6.3.1.1 Reversible ototoxicity

It is known that in humans, aspirin acts as a reversible ototoxin causing temporary loss of absolute hearing sensitivity, alterations of perceived sounds and tinnitus (Cazals, 2000). It would be interesting to test whether the ototoxic effects of this drug are attenuated following washout of the compound. To assess this, measurements could be taken from the same animals following a one hour treatment with aspirin and for a time after washout. Aspirin treatment decreases the number of cells with positive GFP labelling in the *Tg(pou4f3::mGFP)s356t* larvae (Figure 3.7). It will therefore be feasible to assess the animals over time (at regular intervals, up to 10 hours after washout) to examine recovery of the hair cells. It is known that hair cells begin to regenerate in the lateral line 12 hours after initial treatment (Harris et al., 2003; Hernandez et al., 2006). By looking no later than ten hours after treatment has been terminated, it will be possible to rule out any regeneration effects.

#### 6.3.1.2 Prestin

As previously mentioned prestin is a known target of aspirin and has previously been shown to be expressed in zebrafish (Section 6.2.1.2.). It will be interesting to assess the exact expression pattern of the *prestin* gene at 5 dpf and to determine the effect of blocking prestin directly on the appearance and function of the hair cells. The expression pattern of *prestin* could be examined by in situ hybridisation. The inhibitor of protein kinase G, 8-Rp-pCPT-cGMPS, could be applied to block the non-linear capacitance of prestin and to see if the results

mimic those of aspirin treatment (Deak et al., 2005). It would be interesting to discover if prestin is the target for aspirin toxicity in zebrafish hair cells, despite the absence of a cochlea.

#### 6.3.1.3 Overactivation of NMDA receptors

In mammals, it has been suggested that overactivation of NMDA receptors could be a mechanism for aspirin-induced tinnitus (Guitton et al., 2003; Puel and Guitton, 2007). In zebrafish, it could be possible to develop a tinnitus assay by exposing larvae at 5 dpf to white noise and silent gaps using the startle assay rig. The silent stimulus could act as the 'startle-inducer' interrupting the normal swimming behaviour of the larvae. This could be analysed using the Viewpoint tracking software. This assay could then be used to investigate the effects of NMDA receptor antagonists on tinnitus in zebrafish.

Although there is the potential to develop a tinnitus assay, it is worthwhile bearing in mind that NMDA receptor antagonists already have known effects on the central nervous system in zebrafish (Sison and Gerlai, 2011). Specifically, memantine (an NMDA receptor antagonist) increases startle and decreases habituation behaviour (Best et al., 2008).

### 6.3.2 Cisplatin

Cisplatin represents the ideal candidate for a more thorough investigation of the underlying mechanisms of hair cell death in zebrafish using a single compound.

As shown in Chapters 3 and 5, cisplatin is capable of inducing hair cell damage in the zebrafish (also shown in Giari et al., 2012; Ou et al., 2007). Unlike for the aminoglycosides neomycin and gentamicin, there is not a great deal of continuing research regarding the underlying mechanisms of cisplatin-induced hair cell damage in the zebrafish. There is however, a large body of research concerning the mechanisms of this ototoxicity in cell lines and other animal models, as shown above (Section 6.2.1.3).

#### 6.3.2.1 Potential areas for future investigation

There are clearly a number of areas where there is a gap in the research concerning the mechanisms of cisplatin ototoxicity in zebrafish. The table below

highlights areas for further investigation, based on what is known from the literature (Section 6.2.1.3).

**Table 6.1:** Potential areas for further investigation into cisplatin-induced hair cell loss.

| Area | Short-term questions | Long-term questions |
|---|---|---|
| Metabolite effects | Is the hydrolysis metabolite of cisplatin (found in the blood of human patients) sufficient to induce hair cell loss in the pLL? | |
| | How does this damage from the metabolite compare with the damage induced by non-metabolised cisplatin? | |
| Uptake of cisplatin into hair cells | Does OCT2 play a role in the zebrafish in uptake of cisplatin? | Can the timely and spatially controlled knock down of *Ctr1* protect from cisplatin induced hair cell death? |
| | Where are OCT2 and Ctr1 mRNA expressed in the zebrafish? Is it expressed at the time of hair cell loss i.e. 5 dpf? | Can knock down of *OCT2* protect from cisplatin induced hair cell death? |
| Early downstream effects of cisplatin | Does chemical inhibition of the NADPH oxidase family of enzymes protect from cisplatin induced hair cell damage? | Does knock down of components of the NOX family of enzymes protect from cisplatin induced hair cell loss? |
| | Are any components of the NOX family of enzymes expressed at 5 dpf? | |
| | Does chemical inhibition of the zebrafish TRPV1 channel protect from cisplatin induced hair cell damage? | Does knock down of the zebrafish equivalent of *TRPV1* protect from cisplatin induced hair cell loss? |
| | Is the zebrafish equivalent of *TRPV1* mRNA expressed at 5 dpf? | |
| Reactive oxygen species | Can cisplatin exposure induce a quantifiable increase in ROS in zebrafish? | |
| | Which antioxidants confer protection in the pLL against cisplatin induced ototoxicity? What can this tell us about intermediary pathways to cisplatin induced hair cell death? | |
| | Can cisplatin induce $H_2O_2$ in ZF? | |
| Death | Does cisplatin induced hair cell death involve apoptosis alone or some necrosis? | |

6.3.2.2 Future avenues for investigation based on preliminary data

One well-established area of research in non-zebrafish models is the role of reactive oxygen species production in cisplatin-induced hair cell damage (recently reviewed in Casares et al., 2012). The NADPH family of enzymes have been shown to increase reactive oxygen species in response to cisplatin (Banfi et al., 2004; Kim et al., 2010; Mukherjea et al., 2010). Towards the end of the research project, the potential effects of cisplatin on ROS induction were investigated. Preliminary data showed that inhibition of this family of enzymes in particular could confer significant protection from hair cell damage in zebrafish (see Appendix 3). This promising result could form the basis of an independent follow-on project and help to further validate the zebrafish as a translational model for ototoxicity. Specific ideas for future experiments are detailed below.

Gene expression studies

Expression of the NADPH oxidase family of enzymes in the zebrafish could be investigated using in situ hybridisation. The NOX family of enzymes are fairly well conserved compared to humans (Figure 6.2). The Zebrafish genome encodes the NADPH oxidase members *NOX-1*, *-2* and *-4*, *NOX5*-like and *DUOX-1* (Kawahara et al., 2007; Niethammer et al., 2009). *CYBA* (*P22$^{phox}$*) is a key subunit of NOX1-4 in humans and is also present in NOX-1, NOX-2 and NOX-4 in zebrafish; NOX enzyme activity is P22$^{phox}$-dependent. (Bedard and Krause, 2007). Zebrafish lack a NOX-3 equivalent. RNA Probes could be made to the genes *zfCYBA*, *zfNOX-5*-like and *zfDUOX-1*, and the expression pattern assessed between 24 hpf and 5 dpf. Information on the timing of expression of these genes would also be informative if morpholino knockdown were to be performed.

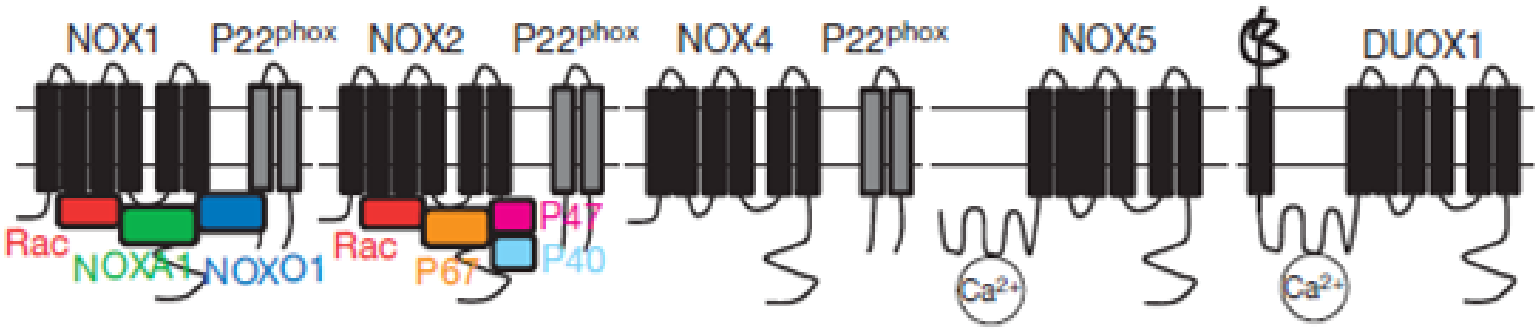



**Figure 6.2: Conserved members of the NADPH oxidase family of enzymes in the zebrafish.** *NOX-1*, *NOX-2* and *NOX-4* genes are dependent on the co-expression of the $P22^{phox}$ (or *CYBA*) subunit. Zebrafish also possess a *NOX-5*-like gene and a *DUOX-1* gene. Zebrafish lack NOX3, which has been proposed to be involved in cisplatin-induced hair cell death in the mammal. Figure taken from Niethammer et al., 2009.

Protection studies

A combination of genetic and compound-based protection studies could be performed in order to determine which of the NADPH oxidase family members is important in perpetuating the hair cell damage cascade.

Specific knockdown of the *CYBA* component of *NOX-1*, *-2* and *-4* and knockdown of *DUOX-1* using splice blocking morpholinos would indicate the importance of these enzymes in the damage process. Morpholino injections could be performed at the 1 cell-stage. The knockdown would be confirmed by PCR from cDNA at days 2, 3, 4 and 5 following fertilisation. The protective effects of knockdown could then be evaluated by the DASPEI assay (blinded to treatment group; as in Section 2.7).

To determine specifically if the NOX-1 enzyme is a key mediator of cisplatin-induced hair cell death, a specific inhibitor (2-acetylphenothiazine) could be applied as a co-treatment with cisplatin and the effects investigated initially with the DASPEI stain (blinded to treatment group; as in Section 2.7; Gianni et al., 2010).

More broadly, tests could be carried out for the downstream effects of cisplatin with or without otoprotectant/morpholino knockdown. For example, an assay using the cell-permeant dye 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) would act as an indicator of reactive oxygen species levels. Furthermore, functional testing (rheotaxis and seeker response test) could be performed to confirm otoprotection.

Additional experiments

Other elements of the pathway of cisplatin-induced damage have the potential to be investigated. It would be informative to establish the uptake mechanism of cisplatin in zebrafish and to determine the role of TRPV1 receptor activation and phosphorylation of STAT transcription factors in eliciting hair cell damage. Again, these pathways could be studied using in situ hybridisation, antibody staining, conditional knockdown and a variety of downstream damage indicators including transgenic lines, the DASPEI assay and functional testing.

## 6.4 Closing remarks

This study described in this thesis has shown that hair cell damage can be induced in larval zebrafish by short-term exposure to a range of known human ototoxins. It is the first study to demonstrate that larval zebrafish can experience deficits in detecting auditory and vibratory stimuli, and in orientation to current flow, following hair cell damage induced by a variety of ototoxic compounds. The findings collectively replicate the histological and functional effects manifested in humans and non-human mammals after exposure to ototoxins, thereby further supporting the use of zebrafish as an early pre-clinical indicator of drug-induced ototoxicity. A number of routes exist for potential future investigation into ototoxicity in the zebrafish. By exploring some of the mechanistic pathways of damage induced by specific compounds and comparing this to what is already known in mammalian and cell line studies, it will be possible to validate the zebrafish fully as an in vivo model of drug-induced hearing and balance impairment.

## References


AAA, 2009. Ototoxicity monitoring. American Academy of Audiology: Position statement and clinical practice guidelines.

Abada, P., Howell, S. B., 2010. Regulation of Cisplatin cytotoxicity by cu influx transporters. Met Based Drugs 2010, 317581.

Abbas, L., Whitfield, T. T., 2009. Nkcc1 (Slc12a2) is required for the regulation of endolymph volume in the otic vesicle and swim bladder volume in the zebrafish larva. Development 136, 2837-2848.

Abbas, L., Whitfield, T. T. 2010. Fish Physiology. Zebrafish, Elsevier. 29**:** 124-160.

Aguilar-Markulis, N. V., Beckley, S., Priore, R., Mettlin, C., 1981. Auditory toxicity effects of long-term cis-dichlorodiammineplatinum II therapy in genitourinary cancer patients. J Surg Oncol 16, 111-123.

AHL. Action on Hearing Loss. 2011, from http://www.actiononhearingloss.org.uk/your-hearing/about-deafness-and-hearing-loss/statistics/adults-who-are-deaf-or-have-a-hearing-loss-in-the-uk.aspx.

AHL, 2011b. Hearing Matters: Taking Action on Hearing Loss in the 21st Century.

Alam, S. A., Robinson, B. K., Huang, J., Green, S. H., 2007. Prosurvival and proapoptotic intracellular signaling in rat spiral ganglion neurons in vivo after the loss of hair cells. J Comp Neurol 503, 832-852.

Albert, J. T., Winter, H., Schaechinger, T. J., Weber, T., Wang, X., He, D. Z., Hendrich, O., Geisler, H. S., Zimmermann, U., Oelmann, K., Knipper, M., Gopfert, M. C., Oliver, D., 2007. Voltage-sensitive prestin orthologue expressed in zebrafish hair cells. J Physiol 580, 451-461.

Alderton, W., Berghmans, S., Butler, P., Chassaing, H., Fleming, A., Golder, Z., Richards, F., Gardner, I., 2010. Accumulation and metabolism of drugs and CYP probe substrates in zebrafish larvae. Xenobiotica 40, 547-557.

Alharazneh, A., Luk, L., Huth, M., Monfared, A., Steyger, P. S., Cheng, A. G., Ricci, A. J., 2011. Functional hair cell mechanotransducer channels are required for aminoglycoside ototoxicity. PLoS One 6, e22347.

Ali, S., Champagne, D. L., Richardson, M. K., 2012. Behavioral profiling of zebrafish embryos exposed to a panel of 60 water-soluble compounds. Behav Brain Res 228, 272-283.

Aman, A., Piotrowski, T., 2008. Wnt/beta-catenin and Fgf signaling control collective cell migration by restricting chemokine receptor expression. Dev Cell 15, 749-761.

Anniko, M., Takada, A., Schacht, J., 1982. Comparative ototoxicities of gentamicin and netilmicin in three model systems. Am J Otolaryngol 3, 422-433.

Anon 1994. Report of an Informal Consultation on Strategies for Prevention of Hearing Impairment from Ototoxic Drugs. Geneva, World Health Organisation.

Anon, 2005. Guidance document on the validation and international acceptance of new or updated test methods for hazard identification. Organisation for Economic Cooperation and Development (OECD). Environmental Health and Safety Monograph Series on Testing and Assessment No. 34., 1–96.

Anon, 2010. FDA Guidance for Industry: M3(R2) nonclinical safety studies for the conduct of human clinical trials and marketing authorization of pharmaceuticals. US Department of Health and Human Services Food and Drug Administration CDER CBER.

Arnold, G. P., 1974. Rheotropism in fishes. Biol. Rev. Camb. Philos. Soc. 49, 515-576.

Arnold, G. P., Weihs, D., 1978. Hydrodynamics of Rheotaxis in Plaice (Pleuronectes-Platessa L). Journal of Experimental Biology 75, 147-&.

Arnold, W., Nadol, J. B., Weidauer, H., 1981. Ultrastructural Histopathology in a Case of Human Ototoxicity Due to Loop Diuretics. Acta Oto-Laryngologica 91, 399-414.

Bae, W. Y., Kim, L. S., Hur, D. Y., Jeong, S. W., Kim, J. R., 2008. Secondary apoptosis of spiral ganglion cells induced by aminoglycoside: Fas-Fas ligand signaling pathway. Laryngoscope 118, 1659-1668.

Baguley, D. M., 2002. Mechanisms of tinnitus. Br Med Bull 63, 195-212.

Baker, C. F., Montgomery, J. C., 1999a. Lateral line mediated rheotaxis in the Antarctic fish Pagothenia borchgrevinki. Polar Biol. 21, 305-309.

Baker, C. F., Montgomery, J. C., 1999b. The sensory basis of rheotaxis in the blind Mexican cave fish, Astyanax fasciatus. J. Comp. Physiol. A Neuroethol. Sens. Neural. Behav. Physiol. 184, 519-527.

Baker, C. F., Montgomery, J. C., 2001. Sensory deficits induced by cadmium in banded kokopu, Galaxias fasciatus, juveniles. Envir. Biol. Fish. 62, 455-464.

Balak, K. J., Corwin, J. T., Jones, J. E., 1990. Regenerated hair cells can originate from supporting cell progeny: evidence from phototoxicity and laser ablation experiments in the lateral line system. J Neurosci 10, 2502-2512.

Banfi, B., Malgrange, B., Knisz, J., Steger, K., Dubois-Dauphin, M., Krause, K. H., 2004. NOX3, a superoxide-generating NADPH oxidase of the inner ear. J Biol Chem 279, 46065-46072.

Bang, P. I., Yelick, P. C., Malicki, J. J., Sewell, W. F., 2002. High-throughput behavioral screening method for detecting auditory response defects in zebrafish. J. Neurosci. Methods 118, 177-187.

Bas, E., Van De Water, T. R., Gupta, C., Dinh, J., Vu, L., Martinez-Soriano, F., Lainez, J. M., Marco, J., 2012. Efficacy of three drugs for protecting against gentamicin-induced hair cell and hearing losses. Br J Pharmacol 166, 1888-1904.

Bauer, C. A., Brozoski, T. J., Rojas, R., Boley, J., Wyder, M., 1999. Behavioral model of chronic tinnitus in rats. Otolaryngol Head Neck Surg 121, 457-462.

Baxendale, S., Holdsworth, C. J., Santoscoy, P. M., Harrison, M. R., Fox, J., Parkin, C. A., Ingham, P. W., Cunliffe, V. T., 2012. Identification of compounds with novel anti-convulsant properties in a zebrafish model of epileptic seizures. Dis Model Mech.

Bedard, K., Krause, K. H., 2007. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 87, 245-313.

Behra, M., Bradsher, J., Sougrat, R., Gallardo, V., Allende, M. L., Burgess, S. M., 2009. Phoenix is required for mechanosensory hair cell regeneration in the zebrafish lateral line. PLoS Genet 5, e1000455.

Behra, M., Gallardo, V. E., Bradsher, J., Torrado, A., Elkahloun, A., Idol, J., Sheehy, J., Zonies, S., Xu, L., Shaw, K. M., Satou, C., Higashijima, S. I., Weinstein, B. M., Burgess, S. M., 2012. Transcriptional signature of accessory cells in the lateral line, using the Tnk1bp1:EGFP transgenic zebrafish line. Bmc Developmental Biology 12.

Bereiter-Hahn, J., 1976. Dimethylaminostyrylmethylpyridiniumiodine (daspmi) as a fluorescent probe for mitochondria in situ. Biochim Biophys Acta 423, 1-14.

Berghmans, S., Butler, P., Goldsmith, P., Waldron, G., Gardner, I., Golder, Z., Richards, F. M., Kimber, G., Roach, A., Alderton, W., Fleming, A., 2008. Zebrafish based assays for the assessment of cardiac, visual and gut function--potential

safety screens for early drug discovery. J Pharmacol Toxicol Methods 58, 59-68.
Berglin, C. E., Pierre, P. V., Bramer, T., Edsman, K., Ehrsson, H., Eksborg, S., Laurell, G., 2011. Prevention of cisplatin-induced hearing loss by administration of a thiosulfate-containing gel to the middle ear in a guinea pig model. Cancer Chemother Pharmacol 68, 1547-1556.
Best, J. D., Berghmans, S., Hunt, J. J., Clarke, S. C., Fleming, A., Goldsmith, P., Roach, A. G., 2008. Non-associative learning in larval zebrafish. Neuropsychopharmacology 33, 1206-1215.
Betz, W. J., Mao, F., Smith, C. B., 1996. Imaging exocytosis and endocytosis. Curr Opin Neurobiol 6, 365-371.
Bever, M. M., Fekete, D. M., 2002. Atlas of the developing inner ear in zebrafish. Dev. Dyn. 223, 536-543.
Blaxter, J. H. S., Fuiman, L. A. 1989. Function of the free neuromasts of marine teleost larvae. The Mechanosensory Lateral Line, Neurobiology and Evolution. Coombs, S, Gorner, P,Munz, H. New York, Springer-Verlag**:** 481-499.
Boheim, K., Bichler, E., 1985. Cisplatin-induced ototoxicity: audiometric findings and experimental cochlear pathology. Arch Otorhinolaryngol 242, 1-6.
Brosamle, C., Halpern, M. E., 2002. Characterization of myelination in the developing zebrafish. Glia 39, 47-57.
Brox, L. W., Gowans, B., Belch, A., 1980. L-phenylalanine mustard (melphalan) uptake and cross-linking in the RPMI 6410 human lymphoblastoid cell line. Cancer Res 40, 1169-1172.
Brunton, L., Lazo, J., Parker, K. 2006. Goodman & Gilman's The Pharmacological Basis of Therapeutics 11th Edition. New York; London, McGraw-Hill.
Buck, L. M., Winter, M. J., Redfern, W. S., Whitfield, T. T., 2012. Ototoxin-induced cellular damage in neuromasts disrupts lateral line function in larval zebrafish. Hear Res 284, 67-81.
Budick, S. A., O'Malley, D. M., 2000. Locomotor repertoire of the larval zebrafish: swimming, turning and prey capture. J Exp Biol 203, 2565-2579.
Burger, H., Zoumaro-Djayoon, A., Boersma, A. W., Helleman, J., Berns, E. M., Mathijssen, R. H., Loos, W. J., Wiemer, E. A., 2010. Differential transport of platinum compounds by the human organic cation transporter hOCT2 (hSLC22A2). Br J Pharmacol 159, 898-908.
Burgess, H. A., Granato, M., 2007. Sensorimotor gating in larval zebrafish. J. Neurosci. 27, 4984-4994.

Burgess, H. A., Granato, M., 2008. The neurogenetic frontier: Lessons from misbehaving zebrafish. Brief. Funct. Genomic. Proteomic. 7, 474-482.

Burgess, H. A., Johnson, S. L., Granato, M., 2009. Unidirectional startle responses and disrupted left-right co-ordination of motor behaviors in robo3 mutant zebrafish. Genes Brain Behav. 8, 500-511.

Burns, C. G., Milan, D. J., Grande, E. J., Rottbauer, W., MacRae, C. A., Fishman, M. C., 2005. High-throughput assay for small molecules that modulate zebrafish embryonic heart rate. Nat Chem Biol 1, 263-264.

Busse, H. J., Wostmann, C., Bakker, E. P., 1992. The bactericidal action of streptomycin: membrane permeabilization caused by the insertion of mistranslated proteins into the cytoplasmic membrane of Escherichia coli and subsequent caging of the antibiotic inside the cells due to degradation of these proteins. J Gen Microbiol 138, 551-561.

Cahill, G. M., Hurd, M. W., Batchelor, M. M., 1998. Circadian rhythmicity in the locomotor activity of larval zebrafish. Neuroreport 9, 3445-3449.

Campbell, K. C., Rybak, L. P., Meech, R. P., Hughes, L., 1996. D-methionine provides excellent protection from cisplatin ototoxicity in the rat. Hear Res 102, 90-98.

Campbell, K. C., Meech, R. P., Rybak, L. P., Hughes, L. F., 1999. D-Methionine protects against cisplatin damage to the stria vascularis. Hear Res 138, 13-28.

Campuzano, S., Vazquez, D., Modolell, J., 1979. Functional interaction of neomycin B and related antibiotics with 30S and 50S ribosomal subunits. Biochem Biophys Res Commun 87, 960-966.

Cao, Y., Semanchik, N., Lee, S. H., Somlo, S., Barbano, P. E., Coifman, R., Sun, Z. X., 2009. Chemical modifier screen identifies HDAC inhibitors as suppressors of PKD models. Proceedings of the National Academy of Sciences of the United States of America 106, 21819-21824.

Carlson, S., Willott, J. F., 1996. The behavioral salience of tones as indicated by prepulse inhibition of the startle response: Relationship to hearing loss and central neural plasticity in C57BL/6J mice. Hearing Research 99, 168-175.

Casares, C., Ramirez-Camacho, R., Trinidad, A., Roldan, A., Jorge, E., Garcia-Berrocal, J. R., 2012. Reactive oxygen species in apoptosis induced by cisplatin: review of physiopathological mechanisms in animal models. Eur Arch Otorhinolaryngol.

Cazals, Y., 2000. Auditory sensori-neural alterations induced by salicylate. Prog Neurobiol 62, 583-631.
Cederroth, C. R., 2012. Loss of aminoglycoside sensitivity in HEI-OC1 cells? Hear Res 292, 83-85.
Chakraborty, C., Nandi, S. S., Sinha, S., Gera, V. K., 2006. Zebrafish caspase-3: molecular cloning, characterization, crystallization and phylogenetic analysis. Protein Pept Lett 13, 633-640.
Chan, P. K., Lin, C. C., Cheng, S. H., 2009. Noninvasive technique for measurement of heartbeat regularity in zebrafish (Danio rerio) embryos. Bmc Biotechnology 9.
Chang, T. Y., Pardo-Martin, C., Allalou, A., Wahlby, C., Yanik, M. F., 2012. Fully automated cellular-resolution vertebrate screening platform with parallel animal processing. Lab Chip 12, 711-716.
Chen, F. Q., Hill, K., Guan, Y. J., Schacht, J., Sha, S. H., 2012. Activation of apoptotic pathways in the absence of cell death in an inner-ear immortomouse cell line. Hear Res 284, 33-41.
Chen, G. D., Kermany, M. H., D'Elia, A., Ralli, M., Tanaka, C., Bielefeld, E. C., Ding, D., Henderson, D., Salvi, R., 2010. Too much of a good thing: long-term treatment with salicylate strengthens outer hair cell function but impairs auditory neural activity. Hear Res 265, 63-69.
Cheng, A. G., Cunningham, L. L., Rubel, E. W., 2003. Hair cell death in the avian basilar papilla: characterization of the in vitro model and caspase activation. J Assoc Res Otolaryngol 4, 91-105.
Chiu, L. L., Cunningham, L. L., Raible, D. W., Rubel, E. W., Ou, H. C., 2008. Using the zebrafish lateral line to screen for ototoxicity. J. Assoc. Res. Otolaryngol. 9, 178-190.
Choi, J., Im, G. J., Chang, J., Chae, S. W., Lee, S. H., Kwon, S. Y., Chung, A. Y., Park, H. C., Jung, H. H., 2011. Protective effects of apocynin on cisplatin-induced ototoxicity in an auditory cell line and in zebrafish. J Appl Toxicol.
Ciarimboli, G., Ludwig, T., Lang, D. F., Pavenstadt, H., Koepsell, H., Piechota, H. J., Haier, J., Jaehde, U., Zisowsky, J., Schlatter, E., 2005. Cisplatin nephrotoxicity is critically mediated via the human organic cation transporter 2. American Journal of Pathology 167, 1477-1484.
Ciarimboli, G., Deuster, D., Knief, A., Sperling, M., Holtkamp, M., Edemir, B., Pavenstadt, H., Lanvers-Kaminsky, C., am Zehnhoff-Dinnesen, A., Schinkel, A. H., Koepsell, H., Jurgens, H., Schlatter, E., 2010. Organic cation transporter 2 mediates

cisplatin-induced oto- and nephrotoxicity and is a target for protective interventions. Am J Pathol 176, 1169-1180.
Ciarimboli, G., 2011. Role of organic cation transporters in drug-induced toxicity. Expert Opin Drug Metab Toxicol 7, 159-174.
Clearwater, S. J., Farag, A. M., Meyer, J. S., 2002. Bioavailability and toxicity of dietborne copper and zinc to fish. Comp Biochem Physiol C Toxicol Pharmacol 132, 269-313.
Clerici, W. J., Hensley, K., DiMartino, D. L., Butterfield, D. A., 1996. Direct detection of ototoxicant-induced reactive oxygen species generation in cochlear explants. Hear Res 98, 116-124.
Coffin, A. B., Reinhart, K. E., Owens, K. N., Raible, D. W., Rubel, E. W., 2009. Extracellular divalent cations modulate aminoglycoside-induced hair cell death in the zebrafish lateral line. Hear Res 253, 42-51.
Coffin, A. B., Ou, H., Owens, K. N., Santos, F., Simon, J. A., Rubel, E. W., Raible, D. W., 2010. Chemical screening for hair cell loss and protection in the zebrafish lateral line. Zebrafish 7, 3-11.
Colwill, R. M., Creton, R., 2011. Imaging escape and avoidance behavior in zebrafish larvae. Rev Neurosci 22, 63-73.
Conlon, B. J., Perry, B. P., Smith, D. W., 1998. Attenuation of neomycin ototoxicity by iron chelation. Laryngoscope 108, 284-287.
Conlon, B. J., Smith, D. W., 1998. Supplemental iron exacerbates aminoglycoside ototoxicity in vivo. Hear Res 115, 1-5.
Conlon, B. J., Aran, J. M., Erre, J. P., Smith, D. W., 1999. Attenuation of aminoglycoside-induced cochlear damage with the metabolic antioxidant alpha-lipoic acid. Hear Res 128, 40-44.
Coombs, S., Montgomery, J. C. 1999. The enigmatic lateral line system. Comparative Hearing: Fishes and Amphibians. Popper, AN,Fay, RR. New York, Springer Handbook of Auditory Research - Springer-Verlag. 11**:** 319-362.
Coombs, S., Braun, C. B., Donovan, B., 2001. The orienting response of Lake Michigan mottled sculpin is mediated by canal neuromasts. J Exp Biol 204, 337-348.
Cooper, L. B., Chan, D. K., Roediger, F. C., Shaffer, B. R., Fraser, J. F., Musatov, S., Selesnick, S. H., Kaplitt, M. G., 2006. AAV-mediated delivery of the caspase inhibitor XIAP protects against cisplatin ototoxicity. Otology & Neurotology 27, 484-490.

Cunningham, L. L., Cheng, A. G., Rubel, E. W., 2002. Caspase activation in hair cells of the mouse utricle exposed to neomycin. J Neurosci 22, 8532-8540.
d'Alençon, C. A., Pena, O. A., Wittmann, C., Gallardo, V. E., Jones, R. A., Loosli, F., Liebel, U., Grabher, C., Allende, M. L., 2010. A high-throughput chemically induced inflammation assay in zebrafish. BMC Biol 8, 151.
Dallos, P., Fakler, B., 2002. Prestin, a new type of motor protein. Nat Rev Mol Cell Biol 3, 104-111.
Dallos, P., 2008. Cochlear amplification, outer hair cells and prestin. Curr Opin Neurobiol 18, 370-376.
Dambly-Chaudiere, C., Cubedo, N., Ghysen, A., 2007. Control of cell migration in the development of the posterior lateral line: antagonistic interactions between the chemokine receptors CXCR4 and CXCR7/RDC1. Bmc Developmental Biology 7.
Dann, O., Bergen, G., Demant, E., Volz, G., 1971. Trypanocide Diamidines of 2-Phenylbenzofuran, 2-Phenylindene and 2-Phenylindole. Annalen Der Chemie-Justus Liebig 749, 68-&.
Darland, T., Dowling, J. E., 2001. Behavioral screening for cocaine sensitivity in mutagenized zebrafish. Proc Natl Acad Sci U S A 98, 11691-11696.
David, N. B., Sapede, D., Saint-Etienne, L., Thisse, C., Thisse, B., Dambly-Chaudiere, C., Rosa, F. M., Ghysen, A., 2002. Molecular basis of cell migration in the fish lateral line: role of the chemokine receptor CXCR4 and of its ligand, SDF1. Proc Natl Acad Sci U S A 99, 16297-16302.
David, R. M., Jones, H. S., Panter, G. H., Winter, M. J., Hutchinson, T. H., Chipman, J. K., 2012. Interference with xenobiotic metabolic activity by the commonly used vehicle solvents dimethylsulfoxide and methanol in zebrafish (Danio rerio) larvae but not Daphnia magna. Chemosphere 88, 912-917.
Davies, J., Gorini, L., Davis, B. D., 1965. Misreading of RNA codewords induced by aminoglycoside antibiotics. Mol Pharmacol 1, 93-106.
Davies, J., Jones, D. S., Khorana, H. G., 1966. A further study of misreading of codons induced by streptomycin and neomycin using ribopolynucleotides containing two nucleotides in alternating sequence as templates. J Mol Biol 18, 48-57.
Davies, J., Davis, B. D., 1968. Misreading of ribonucleic acid code words induced by aminoglycoside antibiotics. The effect of drug concentration. J Biol Chem 243, 3312-3316.
Davis, A., Rafaie, E. A. 2000. Epidemiology of Tinnitus. Tinnitus Handbook. Tyler, RS. San Diego, Singular**:** 1-24.

Davis, A., Moorjani, P. 2002. The Epidemiology of Hearing and Balance Disorders, Cabinet Office

Davis, A. C., 1989. The Prevalence of Hearing Impairment and Reported Hearing Disability among Adults in Great-Britain. International Journal of Epidemiology 18, 911-917.

Davis, B. D., 1987. Mechanism of bactericidal action of aminoglycosides. Microbiol Rev 51, 341-350.

Day, R. O., Graham, G. G., Bieri, D., Brown, M., Cairns, D., Harris, G., Hounsell, J., Platt-Hepworth, S., Reeve, R., Sambrook, P. N., et al., 1989. Concentration-response relationships for salicylate-induced ototoxicity in normal volunteers. Br J Clin Pharmacol 28, 695-702.

De Feo, C. J., Aller, S. G., Siluvai, G. S., Blackburn, N. J., Unger, V. M., 2009. Three-dimensional structure of the human copper transporter hCTR1. Proc Natl Acad Sci U S A 106, 4237-4242.

De Groot, J. C., Huizing, E. H., Veldman, J. E., 1991. Early ultrastructural effects of gentamicin cochleotoxicity. Acta Otolaryngol 111, 273-280.

De Stasio, E. A., Moazed, D., Noller, H. F., Dahlberg, A. E., 1989. Mutations in 16S ribosomal RNA disrupt antibiotic--RNA interactions. EMBO J 8, 1213-1216.

Deak, L., Zheng, J., Orem, A., Du, G. G., Aguinaga, S., Matsuda, K., Dallos, P., 2005. Effects of cyclic nucleotides on the function of prestin. J Physiol 563, 483-496.

Deavall, D. G., Martin, E. A., Horner, J. M., Roberts, R., 2012. Drug-induced oxidative stress and toxicity. J Toxicol 2012, 645460.

Dehne, N., Rauen, U., de Groot, H., Lautermann, J., 2002. Involvement of the mitochondrial permeability transition in gentamicin ototoxicity. Hear Res 169, 47-55.

Delpire, E., Lu, J., England, R., Dull, C., Thorne, T., 1999. Deafness and imbalance associated with inactivation of the secretory Na-K-2Cl co-transporter. Nat Genet 22, 192-195.

Didier, A., Miller, J. M., Nuttall, A. L., 1993. The vascular component of sodium salicylate ototoxicity in the guinea pig. Hear Res 69, 199-206.

Diekmann, H., Hill, A., 2012. ADMETox in Zebrafish. Drug Discov. Today: Dis. Model In Press.

Dijkgraaf, S., 1963. The functioning and significance of the lateral-line organs. Biol. Rev. Camb. Philos. Soc. 38, 51-105.

Ding, D., He, J., Allman, B. L., Yu, D., Jiang, H., Seigel, G. M., Salvi, R. J., 2011. Cisplatin ototoxicity in rat cochlear organotypic cultures. Hear Res 282, 196-203.

Domenici, P., 2010. Context-dependent variability in the components of fish escape response: integrating locomotor performance and behavior. J. Exp. Zool. A Ecol. Genet. Physiol. 313, 59-79.

Dreissen, Y. E., Bakker, M. J., Koelman, J. H., Tijssen, M. A., 2012. Exaggerated startle reactions. Clin Neurophysiol 123, 34-44.

Elks, P. M., van Eeden, F. J., Dixon, G., Wang, X., Reyes-Aldasoro, C. C., Ingham, P. W., Whyte, M. K., Walmsley, S. R., Renshaw, S. A., 2011. Activation of hypoxia-inducible factor-1alpha (Hif-1alpha) delays inflammation resolution by reducing neutrophil apoptosis and reverse migration in a zebrafish inflammation model. Blood 118, 712-722.

Ernest, S., Rauch, G. J., Haffter, P., Geisler, R., Petit, C., Nicolson, T., 2000. Mariner is defective in myosin VIIA: a zebrafish model for human hereditary deafness. Human Molecular Genetics 9, 2189-2196.

Eshraghi, A. A., Wang, J., Adil, E., He, J., Zine, A., Bublik, M., Bonny, C., Puel, J. L., Balkany, T. J., Van de Water, T. R., 2007. Blocking c-Jun-N-terminal kinase signaling can prevent hearing loss induced by both electrode insertion trauma and neomycin ototoxicity. Hearing Research 226, 168-177.

Fang, L. H., Green, S. R., Baek, J. S., Lee, S. H., Ellett, F., Deer, E., Lieschke, G. J., Witztum, J. L., Tsimikas, S., Miller, Y. I., 2011. In vivo visualization and attenuation of oxidized lipid accumulation in hypercholesterolemic zebrafish. Journal of Clinical Investigation 121, 4861-4869.

Faucher, K., Fichet, D., Miramand, P., Lagardere, J. P., 2006. Impact of acute cadmium exposure on the trunk lateral line neuromasts and consequences on the "C-start" response behaviour of the sea bass (Dicentrarchus labrax L.; Teleostei, Moronidae). Aquat. Toxicol. 76, 278-294.

Faucher, K., Fichet, D., Miramand, P., Lagardere, J. P., 2008. Impact of chronic cadmium exposure at environmental dose on escape behaviour in sea bass (Dicentrarchus labrax L.; Teleostei, Moronidae). Environ. Pollut. 151, 148-157.

Faucherre, A., Pujol-Marti, J., Kawakami, K., Lopez-Schier, H., 2009. Afferent Neurons of the Zebrafish Lateral Line Are Strict Selectors of Hair-Cell Orientation. PLoS One 4.

Fausti, S. A., Schechter, M. A., Rappaport, B. Z., Frey, R. H., Mass, R. E., 1984. Early detection of cisplatin ototoxicity. Selected case reports. Cancer 53, 224-231.

Fay, R. R., Popper, A. N., 1974. Acoustic stimulation of the ear of the goldfish (Carassius auratus). J. Exp. Biol. 61, 243-260.

Fay, R. R., Popper, A. N. 1999. Hearing in fishes and amphibians: An introduction. Comparative hearing: fish and amphibians. Popper, AN,Fay, RR. New York, Springer handbook of auditory research- Springer-Verlag. 11**:** 6.

Feghali, J. G., Liu, W., Van De Water, T. R., 2001. L-n-acetyl-cysteine protection against cisplatin-induced auditory neuronal and hair cell toxicity. Laryngoscope 111, 1147-1155.

Fischel-Ghodsian, N. 2004. Genetic Factors in Aminoglycoside Ototototoxicity. Ototoxicity Roland, P,Rutka, J. New York, BC Decker**:** 144-152.

Fleming, A., Alderton, W., 2012. Zebrafish in pharmaceutical industry research: finding the best fit. Drug Discov. Today: Dis. Model. In Press.

Fontaine, E., Lentink, D., Kranenbarg, S., Muller, U. K., van Leeuwen, J. L., Barr, A. H., Burdick, J. W., 2008. Automated visual tracking for studying the ontogeny of zebrafish swimming. J Exp Biol 211, 1305-1316.

Foreman, M. B., Eaton, R. C., 1993. The direction change concept for reticulospinal control of goldfish escape. J. Neurosci. 13, 4101-4113.

Forge, A., Li, L., 2000. Apoptotic death of hair cells in mammalian vestibular sensory epithelia. Hearing Research 139, 97-115.

Fourmy, D., Recht, M. I., Blanchard, S. C., Puglisi, J. D., 1996. Structure of the A site of Escherichia coli 16S ribosomal RNA complexed with an aminoglycoside antibiotic. Science 274, 1367-1371.

Fourmy, D., Recht, M. I., Puglisi, J. D., 1998. Binding of neomycin-class aminoglycoside antibiotics to the A-site of 16 S rRNA. J Mol Biol 277, 347-362.

Gahtan, E., Sankrithi, N., Campos, J. B., O'Malley, D. M., 2002. Evidence for a widespread brain stem escape network in larval zebrafish. Journal of Neurophysiology 87, 608-614.

Gale, J. E., Marcotti, W., Kennedy, H. J., Kros, C. J., Richardson, G. P., 2001. FM1-43 dye behaves as a permeant blocker of the hair-cell mechanotransducer channel. J Neurosci 21, 7013-7025.

Garetz, S. L., Altschuler, R. A., Schacht, J., 1994. Attenuation of gentamicin ototoxicity by glutathione in the guinea pig in vivo. Hear Res 77, 81-87.

Gavrieli, Y., Sherman, Y., Ben-Sasson, S. A., 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119, 493-501.

Genschow, E., Spielmann, H., Scholz, G., Seiler, A., Brown, N., Piersma, A., Brady, M., Clemann, N., Huuskonen, H., Paillard, F., Bremer, S., Becker, K., 2002. The ECVAM international validation study on in vitro embryotoxicity tests: results of the definitive phase and evaluation of prediction models. European Centre for the Validation of Alternative Methods. Altern Lab Anim 30, 151-176.

Ghysen, A., Dambly-Chaudière, C., 2004. Development of the zebrafish lateral line. Curr. Opin. Neurobiol. 14, 67-73.

Ghysen, A., Dambly-Chaudiere, C., 2007. The lateral line microcosmos. Genes & Development 21, 2118-2130.

Gianni, D., Taulet, N., Zhang, H., DerMardirossian, C., Kister, J., Martinez, L., Roush, W. R., Brown, S. J., Bokoch, G. M., Rosen, H., 2010. A novel and specific NADPH oxidase-1 (Nox1) small-molecule inhibitor blocks the formation of functional invadopodia in human colon cancer cells. ACS Chem Biol 5, 981-993.

Giari, L., Dezfuli, B. S., Astolfi, L., Martini, A., 2012. Ultrastructural effects of cisplatin on the inner ear and lateral line system of zebrafish (Danio rerio) larvae. J Appl Toxicol 32, 293-299.

Gibson, F., Walsh, J., Mburu, P., Varela, A., Brown, K. A., Antonio, M., Beisel, K. W., Steel, K. P., Brown, S. D., 1995. A type VII myosin encoded by the mouse deafness gene shaker-1. Nature 374, 62-64.

Gilmour, D., Knaut, H., Maischein, H. M., Nusslein-Volhard, C., 2004. Towing of sensory axons by their migrating target cells in vivo. Nature Neuroscience 7, 491-492.

Gilmour, D. T., Maischein, H. M., Nusslein-Volhard, C., 2002. Migration and function of a glial subtype in the vertebrate peripheral nervous system. Neuron 34, 577-588.

Gompel, N., Cubedo, N., Thisse, C., Thisse, B., Dambly-Chaudiere, C., Ghysen, A., 2001. Pattern formation in the lateral line of zebrafish. Mech Dev 105, 69-77.

Goodyear, R. J., Gale, J. E., Ranatunga, K. M., Kros, C. J., Richardson, G. P., 2008. Aminoglycoside-induced phosphatidylserine externalization in sensory hair cells is regionally restricted, rapid, and reversible. J Neurosci 28, 9939-9952.

Grasl-Kraupp, B., Ruttkay-Nedecky, B., Koudelka, H., Bukowska, K., Bursch, W., Schulte-Hermann, R., 1995. In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology 21, 1465-1468.

Gratton, M. A., Smyth, B. J. 2004. Ototoxicity of Platinum Compounds. Ototoxicity. Roland, P,Rutka, J. New York, BC Decker**:** 60-75.

Grosell, M., Wood, C. M., Walsh, P. J., 2003. Copper homeostasis and toxicity in the elasmobranch Raja erinacea and the teleost Myoxocephalus octodecemspinosus during exposure to elevated water-borne copper. Comp Biochem Physiol C Toxicol Pharmacol 135, 179-190.

Guitton, M. J., Caston, J., Ruel, J., Johnson, R. M., Pujol, R., Puel, J. L., 2003. Salicylate induces tinnitus through activation of cochlear NMDA receptors. J Neurosci 23, 3944-3952.

Gurkov, R., Eshetu, T., Miranda, I. B., Berens-Riha, N., Mamo, Y., Girma, T., Krause, E., Schmidt, M., Hempel, J. M., Loscher, T., 2008. Ototoxicity of artemether/lumefantrine in the treatment of falciparum malaria: a randomized trial. Malar J 7, 179.

Guthrie, O. W., 2008. Aminoglycoside induced ototoxicity. Toxicology 249, 91-96.

Haas, P., Gilmour, D., 2006. Chemokine signaling mediates self-organizing tissue migration in the zebrafish lateral line. Dev Cell 10, 673-680.

Haddon, C., Lewis, J., 1996. Early ear development in the embryo of the zebrafish, Danio rerio. J Comp Neurol 365, 113-128.

Hancock, R. E., Bell, A., 1988. Antibiotic uptake into gram-negative bacteria. Eur J Clin Microbiol Infect Dis 7, 713-720.

Harris, J. A., Cheng, A. G., Cunningham, L. L., MacDonald, G., Raible, D. W., Rubel, E. W., 2003. Neomycin-induced hair cell death and rapid regeneration in the lateral line of zebrafish (Danio rerio). J. Assoc. Res. Otolaryngol. 4, 219-234.

Hashino, E., Shero, M., 1995. Endocytosis of aminoglycoside antibiotics in sensory hair cells. Brain Res 704, 135-140.

Hashino, E., Shero, M., Salvi, R. J., 1997. Lysosomal targeting and accumulation of aminoglycoside antibiotics in sensory hair cells. Brain Res 777, 75-85.

Hassan, E. S., 1986. On the discrimination of spatial intervals by the blind cave fish (Anoptichthys jordani). Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology 159, 701-710.

Hentschel, D. M., Park, K. M., Cilenti, L., Zervos, A. S., Drummond, I., Bonventre, J. V., 2005. Acute renal failure in zebrafish: a novel system to study a complex disease. Am J Physiol Renal Physiol 288, F923-929.

Hentschel, D. M., Mengel, M., Boehme, L., Liebsch, F., Albertin, C., Bonventre, J. V., Haller, H., Schiffer, M., 2007. Rapid

screening of glomerular slit diaphragm integrity in larval zebrafish. Am J Physiol Renal Physiol 293, F1746-1750.
Hernandez, P. P., Moreno, V., Olivari, F. A., Allende, M. L., 2006. Sub-lethal concentrations of waterborne copper are toxic to lateral line neuromasts in zebrafish (Danio rerio). Hear. Res. 213, 1-10.
Hernandez, P. P., Olivari, F. A., Sarrazin, A. F., Sandoval, P. C., Allende, M. L., 2007. Regeneration in zebrafish lateral line neuromasts: expression of the neural progenitor cell marker sox2 and proliferation-dependent and-independent mechanisms of hair cell renewal. Dev Neurobiol 67, 637-654.
Hinshaw, H. C., Feldman, W. H., 1945. Streptomycin in Treatment of Clinical Tuberculosis - a Preliminary Report. Proceedings of the Staff Meetings of the Mayo Clinic 20, 313-318.
Hirose, K., Hockenbery, D. M., Rubel, E. W., 1997. Reactive oxygen species in chick hair cells after gentamicin exposure in vitro. Hear Res 104, 1-14.
Hirose, K., Westrum, L. E., Cunningham, D. E., Rubel, E. W., 2004. Electron microscopy of degenerative changes in the chick basilar papilla after gentamicin exposure. J Comp Neurol 470, 164-180.
Hirose, Y., Simon, J. A., Ou, H. C., 2011. Hair Cell Toxicity in Anti-cancer Drugs: Evaluating an Anti-cancer Drug Library for Independent and Synergistic Toxic Effects on Hair Cells Using the Zebrafish Lateral Line. J Assoc Res Otolaryngol 6.
Holmes, S., Padgham, N. D., 2011. "Ringing in the Ears": Narrative Review of Tinnitus and Its Impact. Biological Research for Nursing 13, 97-108.
Homma, K., Dallos, P., 2011. Evidence That Prestin Has at Least Two Voltage-dependent Steps. Journal of Biological Chemistry 286, 2297-2307.
Hong, S. H., Park, S. K., Cho, Y. S., Lee, H. S., Kim, K. R., Kim, M. G., Chung, W. H., 2006. Gentamicin induced nitric oxide-related oxidative damages on vestibular afferents in the guinea pig. Hear Res 211, 46-53.
Horlington, 1968. A Method for Measuring Acoustic Startle Response Latency and Magnitude in Rats - Detection of a Single Stimulus Effect Using Latency Measurements. Physiology & Behavior 3, 839-&.
Hu, D. N., Qui, W. Q., Wu, B. T., Fang, L. Z., Zhou, F., Gu, Y. P., Zhang, Q. H., Yan, J. H., Ding, Y. Q., Wong, H., 1991. Genetic aspects of antibiotic induced deafness: mitochondrial inheritance. Journal of Medical Genetics 28, 79-83.

Huizing, E. H., de Groot, J. C., 1987. Human cochlear pathology in aminoglycoside ototoxicity--a review. Acta Otolaryngol Suppl 436, 117-125.

Human, H., Hagen, C. M., de Jong, G., Harris, T., Lombard, D., Christiansen, M., Bardien, S., 2010. Investigation of mitochondrial sequence variants associated with aminoglycoside-induced ototoxicity in South African TB patients on aminoglycosides. Biochem Biophys Res Commun 393, 751-756.

Hurd, M. W., Debruyne, J., Straume, M., Cahill, G. M., 1998. Circadian rhythms of locomotor activity in zebrafish. Physiol Behav 65, 465-472.

Hutchinson, T. H., Bogi, C., Winter, M. J., Owens, J. W., 2009. Benefits of the maximum tolerated dose (MTD) and maximum tolerated concentration (MTC) concept in aquatic toxicology. Aquat Toxicol 91, 197-202.

ICH, 2005. ICH S7A: Safety pharmacology studies for human pharmaceuticals.

Ikeda, K., Oshima, T., Hidaka, H., Takasaka, T., 1997. Molecular and clinical implications of loop diuretic ototoxicity. Hearing Research 107, 1-8.

Issa, F. A., O'Brien, G., Kettunen, P., Sagasti, A., Glanzman, D. L., Papazian, D. M., 2011. Neural circuit activity in freely behaving zebrafish (Danio rerio). J Exp Biol 214, 1028-1038.

Itoh, M., Chitnis, A. B., 2001. Expression of proneural and neurogenic genes in the zebrafish lateral line primordium correlates with selection of hair cell fate in neuromasts. Mech Dev 102, 263-266.

Jaspers, R. M. A., Muijser, H., Lammers, J. H. C. M., Kulig, B. M., 1993. Mid-Frequency Hearing-Loss and Reduction of Acoustic Startle Responding in Rats Following Trichloroethylene Exposure. Neurotoxicology and Teratology 15, 407-412.

Jastreboff, P. J., Brennan, J. F., Coleman, J. K., Sasaki, C. T., 1988. Phantom auditory sensation in rats: an animal model for tinnitus. Behav Neurosci 102, 811-822.

Jat, P. S., Sharp, P. A., 1989. Cell lines established by a temperature-sensitive simian virus 40 large-T-antigen gene are growth restricted at the nonpermissive temperature. Mol Cell Biol 9, 1672-1681.

Jat, P. S., Noble, M. D., Ataliotis, P., Tanaka, Y., Yannoutsos, N., Larsen, L., Kioussis, D., 1991. Direct derivation of conditionally immortal cell lines from an H-2Kb-tsA58 transgenic mouse. Proc Natl Acad Sci U S A 88, 5096-5100.

Jeong, S. W., Kim, L. S., Hur, D., Bae, W. Y., Kim, J. R., Lee, J. H., 2010. Gentamicin-induced spiral ganglion cell death: apoptosis mediated by ROS and the JNK signaling pathway. Acta Otolaryngol 130, 670-678.

Jette, C. A., Flanagan, A. M., Ryan, J., Pyati, U. J., Carbonneau, S., Stewart, R. A., Langenau, D. M., Look, A. T., Letai, A., 2008. BIM and other BCL-2 family proteins exhibit cross-species conservation of function between zebrafish and mammals. Cell Death and Differentiation 15, 1063-1072.

Jiang, H., Sha, S. H., Forge, A., Schacht, J., 2006. Caspase-independent pathways of hair cell death induced by kanamycin in vivo. Cell Death Differ 13, 20-30.

Jiang, X., Li, W., Zang, H., Wang, J., Guan, C., Yang, N., 2005. [Apoptosis and its molecular mechanism in vestibular hair cell after gentamycin toxicity]. Lin Chuang Er Bi Yan Hou Ke Za Zhi 19, 886-889.

Johnson, A., Carew, E., Sloman, K. A., 2007. The effects of copper on the morphological and functional development of zebrafish embryos. Aquat. Toxicol. 84, 431-438.

Joint Formulary Committee 2011. British National Formulary. Joint Formulary Committee. London, British Medical Association and Royal Pharmaceutical Society.

Jones, H. S., Panter, G. H., Hutchinson, T. H., Chipman, J. K., 2010. Oxidative and conjugative xenobiotic metabolism in zebrafish larvae in vivo. Zebrafish 7, 23-30.

Jones, H. S., Trollope, H. T., Hutchinson, T. H., Panter, G. H., Chipman, J. K., 2012. Metabolism of ibuprofen in zebrafish larvae. Xenobiotica.

Jones, K. S., Alimov, A. P., Rilo, H. L., Jandacek, R. J., Woollett, L. A., Penberthy, W. T., 2008. A high throughput live transparent animal bioassay to identify non-toxic small molecules or genes that regulate vertebrate fat metabolism for obesity drug development. Nutrition & Metabolism 5.

Kakehata, S., Santos-Sacchi, J., 1996. Effects of salicylate and lanthanides on outer hair cell motility and associated gating charge. J Neurosci 16, 4881-4889.

Kalinec, F., Kalinec, G., Boukhvalova, M., Kachar, B., 1999. Establishment and characterization of conditionally immortalized organ of corti cell lines. Cell Biol Int 23, 175-184.

Kalinec, G. M., Fernandez-Zapico, M. E., Urrutia, R., Esteban-Cruciani, N., Chen, S., Kalinec, F., 2005. Pivotal role of Harakiri in the induction and prevention of gentamicin-induced hearing loss. Proc Natl Acad Sci U S A 102, 16019-16024.

Kane, D. A., Hammerschmidt, M., Mullins, M. C., Maischein, H. M., Brand, M., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Haffter, P., Heisenberg, C. P., Jiang, Y. J., Kelsh, R. N., Odenthal, J., Warga, R. M., Nusslein-Volhard, C., 1996. The zebrafish epiboly mutants. Development 123, 47-55.

Karasawa, T., Wang, Q., Fu, Y., Cohen, D. M., Steyger, P. S., 2008. TRPV4 enhances the cellular uptake of aminoglycoside antibiotics. J Cell Sci 121, 2871-2879.

Karasawa, T., Wang, Q., David, L. L., Steyger, P. S., 2010. CLIMP-63 is a gentamicin-binding protein that is involved in drug-induced cytotoxicity. Cell Death Dis 1, e102.

Karasawa, T., Steyger, P. S., 2011. Intracellular mechanisms of aminoglycoside-induced cytotoxicity. Integr Biol (Camb) 3, 879-886.

Kaur, T., Mukherjea, D., Sheehan, K., Jajoo, S., Rybak, L. P., Ramkumar, V., 2011. Short interfering RNA against STAT1 attenuates cisplatin-induced ototoxicity in the rat by suppressing inflammation. Cell Death Dis 2, e180.

Kaus, S., 1987. The Effect of Aminoglycoside Antibiotics on the Lateral Line Organ of Aplocheilus lineatus (Cyprinodontidae). Acta. Otolaryngol. 103, 291-298.

Kaus, S., 1992. The influence of calcium on the ototoxicity of aminoglycosides. Acta Otolaryngol 112, 83-87.

Kawahara, T., Quinn, M. T., Lambeth, J. D., 2007. Molecular evolution of the reactive oxygen-generating NADPH oxidase (Nox/Duox) family of enzymes. BMC Evol Biol 7, 109.

Kelly, T. C., Whitworth, C. A., Husain, K., Rybak, L. P., 2003. Aminoguanidine reduces cisplatin ototoxicity. Hear Res 186, 10-16.

Kil, J., Warchol, M. E., Corwin, J. T., 1997. Cell death, cell proliferation, and estimates of hair cell life spans in the vestibular organs of chicks. Hear Res 114, 117-126.

Kim, C. H., Kang, S. U., Pyun, J., Lee, M. H., Hwang, H. S., Lee, H., 2008. Epicatechin protects auditory cells against cisplatin-induced death. Apoptosis 13, 1184-1194.

Kim, H. J., Lee, J. H., Kim, S. J., Oh, G. S., Moon, H. D., Kwon, K. B., Park, C., Park, B. H., Lee, H. K., Chung, S. Y., Park, R., So, H. S., 2010. Roles of NADPH oxidases in cisplatin-induced reactive oxygen species generation and ototoxicity. J Neurosci 30, 3933-3946.

Kim, H. J., Oh, G. S., Lee, J. H., Lyu, A. R., Ji, H. M., Lee, S. H., Song, J., Park, S. J., You, Y. O., Sul, J. D., Park, C., Chung, S. Y., Moon, S. K., Lim, D. J., So, H. S., Park, R., 2011. Cisplatin

ototoxicity involves cytokines and STAT6 signaling network. Cell Res 21, 944-956.
Kimmel, C. B., Patterson, J., Kimmel, R. O., 1974. The development and behavioral characteristics of the startle response in the zebra fish. Dev. Psychobiol. 7, 47-60.
Kimmel, C. B., Eaton, K. B., Powell, S. L., 1980. Decreased fast-start performance of zebrafish lacking Mauthner neurons. J. Comp. Physiol. A Neuroethol. Sens. Neural. Behav. Physiol. 140, 343-350.
Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., Schilling, T. F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253-310.
Kindt, K. S., Finch, G., Nicolson, T., 2012. Kinocilia mediate mechanosensitivity in developing zebrafish hair cells. Dev Cell 23, 329-341.
Koch, M., 1999. The neurobiology of startle. Prog Neurobiol 59, 107-128.
Kohashi, T., Oda, Y., 2008. Initiation of Mauthner- or non-Mauthner-mediated fast escape evoked by different modes of sensory input. J Neurosci 28, 10641-10653.
Kokel, D., Bryan, J., Laggner, C., White, R., Cheung, C. Y., Mateus, R., Healey, D., Kim, S., Werdich, A. A., Haggarty, S. J., Macrae, C. A., Shoichet, B., Peterson, R. T., 2010. Rapid behavior-based identification of neuroactive small molecules in the zebrafish. Nat Chem Biol 6, 231-237.
Konrad-Martin, D., 2005. Audiological monitoring of patients receiving ototoxic drugs. ASHA Special Interest Division 6 Perspectives on Hearing and Hearing Disorders: Research And Diagnosis 9 2-10.
Kopke, R. D., Liu, W., Gabaizadeh, R., Jacono, A., Feghali, J., Spray, D., Garcia, P., Steinman, H., Malgrange, B., Ruben, R. J., Rybak, L., VandeWater, T. R., 1997. Use of organotypic cultures of Corti's organ to study the protective effects of antioxidant molecules on cisplatin-induced damage of auditory hair cells. American Journal of Otology 18, 559-571.
Kossl, M., Richardson, G. P., Russell, I. J., 1990. Stereocilia bundle stiffness: effects of neomycin sulphate, A23187 and concanavalin A. Hear Res 44, 217-229.
Kotecha, B., Richardson, G. P., 1994. Ototoxicity in vitro: effects of neomycin, gentamicin, dihydrostreptomycin, amikacin, spectinomycin, neamine, spermine and poly-L-lysine. Hear Res 73, 173-184.

Kubista, M., Akerman, B., Norden, B., 1987. Characterization of interaction between DNA and 4',6-diamidino-2-phenylindole by optical spectroscopy. Biochemistry 26, 4545-4553.

Ladher, R. K., O'Neill, P., Begbie, J., 2010. From shared lineage to distinct functions: the development of the inner ear and epibranchial placodes. Development 137, 1777-1785.

Lambert, F. M., Beck, J. C., Baker, R., Straka, H., 2008. Semicircular canal size determines the developmental onset of angular vestibuloocular reflexes in larval Xenopus. Journal of Neuroscience 28, 8086-8095.

Landis, C., Hunt, W. A. 1939. The startle pattern. New York, Farrar and Rinehart.

Lang, H., Liu, C., 1997. Apoptosis and hair cell degeneration in the vestibular sensory epithelia of the guinea pig following a gentamicin insult. Hear Res 111, 177-184.

Langheinrich, U., Vacun, G., Wagner, T., 2003. Zebrafish embryos express an orthologue of HERG and are sensitive toward a range of QT-prolonging drugs inducing severe arrhythmia. Toxicol Appl Pharmacol 193, 370-382.

Lawlor, P., Marcotti, W., Rivolta, M. N., Kros, C. J., Holley, M. C., 1999. Differentiation of mammalian vestibular hair cells from conditionally immortal, postnatal supporting cells. J Neurosci 19, 9445-9458.

Lecaudey, V., Cakan-Akdogan, G., Norton, W. H., Gilmour, D., 2008. Dynamic Fgf signaling couples morphogenesis and migration in the zebrafish lateral line primordium. Development 135, 2695-2705.

Lee, C. K., Shin, J. I., Cho, Y. S., 2011. Protective Effect of Minocycline Against Cisplatin-induced Ototoxicity. Clin Exp Otorhinolaryngol 4, 77-82.

Lee, J. E., Nakagawa, T., Kim, T. S., Iguchi, F., Endo, T., Kita, T., Murai, N., Naito, Y., Lee, S. H., Ito, J., 2004a. Signaling pathway for apoptosis of vestibular hair cells of mice due to aminoglycosides. Acta Otolaryngol Suppl, 69-74.

Lee, J. S., Kang, S. U., Hwang, H. S., Pyun, J. H., Choung, Y. H., Kim, C. H., 2010. Epicatechin protects the auditory organ by attenuating cisplatin-induced ototoxicity through inhibition of ERK. Toxicology Letters 199, 308-316.

Lee, N. S., Byun, J. H., Bae, S. B., Kim, C. K., Lee, K. T., Park, S. K., Won, J. H., Hong, D. S., Park, H. S., 2004b. Combination of gemcitabine and cisplatin as first-line therapy in advanced non-small-cell lung cancer. Cancer Res. Treat. 36, 173-177.

Lessman, C. A., 2011. The developing zebrafish (Danio rerio): a vertebrate model for high-throughput screening of chemical libraries. Birth Defects Res C Embryo Today 93, 268-280.
Li, L., Nevill, G., Forge, A., 1995. Two modes of hair cell loss from the vestibular sensory epithelia of the guinea pig inner ear. J Comp Neurol 355, 405-417.
Li, Q., Shirabe, K., Kuwada, J. Y., 2004. Chemokine signaling regulates sensory cell migration in zebrafish. Dev Biol 269, 123-136.
Li, Y., Huang, W., Huang, S., Du, J., Huang, C., 2012. Screening of anti-cancer agent using zebrafish: comparison with the MTT assay. Biochem Biophys Res Commun 422, 85-90.
Liang, F., Schulte, B. A., Qu, C., Hu, W., Shen, Z., 2005. Inhibition of the calcium- and voltage-dependent big conductance potassium channel ameliorates cisplatin-induced apoptosis in spiral ligament fibrocytes of the cochlea. Neuroscience 135, 263-271.
Linbo, T. L., Stehr, C. M., Incardona, J. P., Scholz, N. L., 2006. Dissolved copper triggers cell death in the peripheral mechanosensory system of larval fish. Environ. Toxicol. Chem. 25, 597-603.
Liu, K. S., Fetcho, J. R., 1999. Laser ablations reveal functional relationships of segmental hindbrain neurons in zebrafish. Neuron 23, 325-335.
Liu, K. S., Gray, M., Otto, S. J., Fetcho, J. R., Beattie, C. E., 2003. Mutations in deadly seven/notch1a reveal developmental plasticity in the escape response circuit. J Neurosci 23, 8159-8166.
Liu, S., Leach, S. D., 2011. Zebrafish Models for Cancer. Annual Review of Pathology: Mechanisms of Disease, Vol 6 6, 71-93.
Liu, Y. C., Bailey, I., Hale, M. E., 2012. Alternative startle motor patterns and behaviors in the larval zebrafish (Danio rerio). J Comp Physiol A Neuroethol Sens Neural Behav Physiol 198, 11-24.
Lodhi, S., Weiner, N. D., Mechigian, I., Schacht, J., 1980. Ototoxicity of aminoglycosides correlated with their action on monomolecular films of polyphosphoinositides. Biochem Pharmacol 29, 597-601.
Loynes, C. A., Martin, J. S., Robertson, A., Trushell, D. M., Ingham, P. W., Whyte, M. K., Renshaw, S. A., 2010. Pivotal Advance: Pharmacological manipulation of inflammation resolution during spontaneously resolving tissue neutrophilia in the zebrafish. J Leukoc Biol 87, 203-212.

Lu, J., Tsai, T., Choo, S., Yeh, S., Tang, R., Yang, A., Lee, H., 2011. Induction of apoptosis and inhibition of cell growth by tbx5 knockdown contribute to dysmorphogenesis in Zebrafish embryos. J Biomed Sci 18, 73.

Maher, J. F., Schreiner, G. E., 1965. Studies on Ethacrynic Acid in Patients with Refractory Edema. Ann Intern Med 62, 15-29.

Malgrange, B., Lefebvre, P. P., van de Water, T. R., Bonnet, C., Monville, F., Rigo, J. M., Staecker, H., Moonen, G., 1998. In Vitro Ototoxicity of aminoglycosides and platin derivatives. A semi-automatic assay for sensory hair cell damage in explanted rat organ of corti. Toxicol In Vitro 12, 705-714.

Marcotti, W., van Netten, S. M., Kros, C. J., 2005. The aminoglycoside antibiotic dihydrostreptomycin rapidly enters mouse outer hair cells through the mechano-electrical transducer channels. J Physiol 567, 505-521.

Marshall, W. S., Nishioka, R. S., 1980. Relation of Mitochondria-Rich Chloride Cells to Active Chloride Transport in the Skin of a Marine Teleost. Journal of Experimental Zoology 214, 147-156.

Mathers, C., Boerma, T., Ma Fat, D., 2008. The Global Burden of Disease: 2004 Update. World Health Organisation.

Mathers, C. D., Loncar, D., 2006. Projections of global mortality and burden of disease from 2002 to 2030. PLoS Med 3, e442.

Matsui, J. I., Haque, A., Huss, D., Messana, E. P., Alosi, J. A., Roberson, D. W., Cotanche, D. A., Dickman, J. D., Warchol, M. E., 2003. Caspase inhibitors promote vestibular hair cell survival and function after aminoglycoside treatment in vivo. J Neurosci 23, 6111-6122.

Matsuura, S., Ikeda, K., Furukawa, T., 1971. Effects of streptomycin, kanamycin, quinine, and other drugs on the microphonic potentials of goldfish sacculus. Jpn. J. Physiol. 21, 579-590.

Mazurek, B., Lou, X., Olze, H., Haupt, H., Szczepek, A. J., 2012. In vitro protection of auditory hair cells by salicylate from the gentamicin-induced but not neomycin-induced cell loss. Neurosci Lett 506, 107-110.

McDermott, B. M., Jr., Asai, Y., Baucom, J. M., Jani, S. D., Castellanos, Y., Gomez, G., McClintock, J. M., Starr, C. J., Hudspeth, A. J., 2010. Transgenic labeling of hair cells in the zebrafish acousticolateralis system. Gene Expr Patterns 10, 113-118.

McFadden, S. L., Ding, D., Salvemini, D., Salvi, R. J., 2003. M40403, a superoxide dismutase mimetic, protects cochlear

hair cells from gentamicin, but not cisplatin toxicity. Toxicol Appl Pharmacol 186, 46-54.
McHenry, M. J., Strother, J. A., van Netten, S. M., 2008. Mechanical filtering by the boundary layer and fluid-structure interaction in the superficial neuromast of the fish lateral line system. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 194, 795-810.
McHenry, M. J., Feitl, K. E., Strother, J. A., Van Trump, W. J., 2009. Larval zebrafish rapidly sense the water flow of a predator's strike. Biol. Lett. 5, 477-479.
Metcalfe, W. K., Kimmel, C. B., Schabtach, E., 1985. Anatomy of the posterior lateral line system in young larvae of the zebrafish. J. Comp. Neurol. 233, 377-389.
Metcalfe, W. K., 1985. Sensory Neuron Growth Cones Comigrate with Posterior Lateral Line Primordial Cells in Zebrafish. J. Comp. Neurol. 238, 218-224.
Metcalfe, W. K. 1989. Organisation and development of the zebrafish posterior lateral line. The mechanosensory lateral line: neurobiology and evolution. Coombs, S, Gorner, P,Munz, H. New York, Springer-Verlag**:** 147-159.
Meyers, J. R., MacDonald, R. B., Duggan, A., Lenzi, D., Standaert, D. G., Corwin, J. T., Corey, D. P., 2003. Lighting up the senses: FM1-43 loading of sensory cells through nonselective ion channels. J Neurosci 23, 4054-4065.
Mini, E., Nobili, S., Caciagli, B., Landini, I., Mazzei, T., 2006. Cellular pharmacology of gemcitabine. Ann Oncol 17 Suppl 5, v7-12.
Misumi, M., Nishimura, T., Komai, T., Tanaka, N., 1978. Interaction of kanamycin and related antibiotics with the large subunit of ribosomes and the inhibition of translocation. Biochem Biophys Res Commun 84, 358-365.
Moazed, D., Noller, H. F., 1987. Interaction of antibiotics with functional sites in 16S ribosomal RNA. Nature 327, 389-394.
Monsell, E. M. 2007. Nonhereditary Hearing Impairment. Clinical Otology. Hughes, GB,Pensak, ML, Thieme Medical Publishers**:** 306.
Montgomery, J., Coombs, S., Halstead, M., 1995. Biology of the Mechanosensory Lateral-Line in Fishes. Reviews in Fish Biology and Fisheries 5, 399-416.
Montgomery, J. C., Baker, C. F., Carton, A. G., 1997. The lateral line can mediate rheotaxis in fish. Nature 389, 960-963.
Montie, T., Patamasucon, P., 1995. Aminoglycosides: the complex problem of antibiotic mechanisms and clinical applications. Eur J Clin Microbiol Infect Dis 14, 85-87.

Moon, I. S., So, J. H., Jung, Y. M., Lee, W. S., Kim, E. Y., Choi, J. H., Kim, C. H., Choi, J. Y., 2011. Fucoidan promotes mechanosensory hair cell regeneration following amino glycoside-induced cell death. Hear Res 282, 236-242.

More, S. S., Akil, O., Ianculescu, A. G., Geier, E. G., Lustig, L. R., Giacomini, K. M., 2010. Role of the copper transporter, CTR1, in platinum-induced ototoxicity. J Neurosci 30, 9500-9509.

Mu, M. Y., Pao, X., Jie, T., Yu, Y. J., 1996. The alteration of ultrastructure and immunoreactivity of human embryonic organ of Corti tissue culture after exposure to aminoglycoside (neomycin) ototoxicity. Acta Otolaryngol 116, 799-804.

Mukherjea, D., Whitworth, C. A., Nandish, S., Dunaway, G. A., Rybak, L. P., Ramkumar, V., 2006. Expression of the kidney injury molecule 1 in the rat cochlea and induction by cisplatin. Neuroscience 139, 733-740.

Mukherjea, D., Jajoo, S., Whitworth, C., Bunch, J. R., Turner, J. G., Rybak, L. P., Ramkumar, V., 2008. Short interfering RNA against transient receptor potential vanilloid 1 attenuates cisplatin-induced hearing loss in the rat. J Neurosci 28, 13056-13065.

Mukherjea, D., Jajoo, S., Kaur, T., Sheehan, K. E., Ramkumar, V., Rybak, L. P., 2010. Transtympanic administration of short interfering (si)RNA for the NOX3 isoform of NADPH oxidase protects against cisplatin-induced hearing loss in the rat. Antioxid Redox Signal 13, 589-598.

Mukherjea, D., Jajoo, S., Sheehan, K., Kaur, T., Sheth, S., Bunch, J., Perro, C., Rybak, L. P., Ramkumar, V., 2011. NOX3 NADPH oxidase couples transient receptor potential vanilloid 1 to signal transducer and activator of transcription 1-mediated inflammation and hearing loss. Antioxid Redox Signal 14, 999-1010.

Muller, U. K., van Leeuwen, J. L., 2004. Swimming of larval zebrafish: ontogeny of body waves and implications for locomotory development. J. Exp. Biol. 207, 853-868.

Murakami, S. L., Cunningham, L. L., Werner, L. A., Bauer, E., Pujol, R., Raible, D. W., Rubel, E. W., 2003. Developmental differences in susceptibility to neomycin-induced hair cell death in the lateral line neuromasts of zebrafish (Danio rerio). Hear. Res. 186, 47-56.

Muto, A., Orger, M. B., Wehman, A. M., Smear, M. C., Kay, J. N., Page-McCaw, P. S., Gahtan, E., Xiao, T., Nevin, L. M., Gosse, N. J., Staub, W., Finger-Baier, K., Baier, H., 2005. Forward

genetic analysis of visual behavior in zebrafish. Plos Genetics 1, 575-588.
Nakagawa, T., Yamane, H., Shibata, S., Sunami, K., Nakai, Y., 1997. Cell death caused by the acute effects of aminoglycoside and zinc in the ampullary cristae of guinea pigs. Eur Arch Otorhinolaryngol 254, 153-157.
Nakagawa, T., Yamane, H., Takayama, M., Sunami, K., Nakai, Y., 1998. Apoptosis of guinea pig cochlear hair cells following chronic aminoglycoside treatment. Eur Arch Otorhinolaryngol 255, 127-131.
Nakamagoe, M., Tabuchi, K., Uemaetomari, I., Nishimura, B., Hara, A., 2010. Estradiol protects the cochlea against gentamicin ototoxicity through inhibition of the JNK pathway. Hear Res 261, 67-74.
Namdaran, P., Reinhart, K. E., Owens, K. N., Raible, D. W., Rubel, E. W., 2012. Identification of modulators of hair cell regeneration in the zebrafish lateral line. J Neurosci 32, 3516-3528.
Nechiporuk, A., Raible, D. W., 2008. FGF-dependent mechanosensory organ patterning in zebrafish. Science 320, 1774-1777.
Neumeister, H., Szabo, T. M., Preuss, T., 2008. Behavioral and physiological characterization of sensorimotor gating in the goldfish startle response. Journal of Neurophysiology 99, 1493-1502.
Nicolson, T., Rusch, A., Friedrich, R. W., Granato, M., Ruppersberg, J. P., Nusslein-Volhard, C., 1998. Genetic analysis of vertebrate sensory hair cell mechanosensation: the zebrafish circler mutants. Neuron 20, 271-283.
Niethammer, P., Grabher, C., Look, A. T., Mitchison, T. J., 2009. A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. Nature 459, 996-999.
NIH. Retrieved 4/9/2012, from http://www.nih.gov/science/models/zebrafish/.
Ninkovic, J., Bally-Cuif, L., 2006. The zebrafish as a model system for assessing the reinforcing properties of drugs of abuse. Methods 39, 262-274.
Nishikawa, S., Sasaki, F., 1996. Internalization of styryl dye FM1-43 in the hair cells of lateral line organs in Xenopus larvae. Journal of Histochemistry & Cytochemistry 44, 733-741.
Nüsslein-Volhard, C., Dahm, R. 2002. Zebrafish: A Practical Approach. Hames, BD. Oxford, Oxford University Press.

Okuda, M., Saito, H., Urakami, Y., Takano, M., Inui, K., 1996. cDNA cloning and functional expression of a novel rat kidney organic cation transporter, OCT2. Biochem Biophys Res Commun 224, 500-507.

Okuda, T., Sugahara, K., Takemoto, T., Shimogori, H., Yamashita, H., 2005. Inhibition of caspases alleviates gentamicin-induced cochlear damage in guinea pigs. Auris Nasus Larynx 32, 33-37.

Oldenburg, J., Kraggerud, S. M., Brydoy, M., Cvancarova, M., Lothe, R. A., Fossa, S. D., 2007a. Association between long-term neuro-toxicities in testicular cancer survivors and polymorphisms in glutathione-s-transferase-P1 and -M1, a retrospective cross sectional study. J Transl Med 5, 70.

Oldenburg, J., Kraggerud, S. M., Cvancarova, M., Lothe, R. A., Fossa, S. D., 2007b. Cisplatin-induced long-term hearing impairment is associated with specific glutathione s-transferase genotypes in testicular cancer survivors. J Clin Oncol 25, 708-714.

Olivari, F. A., Hernandez, P. P., Allende, M. L., 2008. Acute copper exposure induces oxidative stress and cell death in lateral line hair cells of zebrafish larvae. Brain. Res. 1244, 1-12.

Oliver, D., He, D. Z., Klocker, N., Ludwig, J., Schulte, U., Waldegger, S., Ruppersberg, J. P., Dallos, P., Fakler, B., 2001. Intracellular anions as the voltage sensor of prestin, the outer hair cell motor protein. Science 292, 2340-2343.

Olszewski, J., Haehnel, M., Taguchi, M., Liao, J. C., 2012. Zebrafish larvae exhibit rheotaxis and can escape a continuous suction source using their lateral line. PLoS One 7, e36661.

Ou, H. C., Raible, D. W., Rubel, E. W., 2007. Cisplatin-induced hair cell loss in zebrafish (Danio rerio) lateral line. Hear. Res. 233, 46-53.

Ou, H. C., Cunningham, L. L., Francis, S. P., Brandon, C. S., Simon, J. A., Raible, D. W., Rubel, E. W., 2009. Identification of FDA-approved drugs and bioactives that protect hair cells in the zebrafish (Danio rerio) lateral line and mouse (Mus musculus) utricle. J. Assoc. Res. Otolaryngol. 10, 191-203.

Ou, H. C., Santos, F., Raible, D. W., Simon, J. A., Rubel, E. W., 2010. Drug screening for hearing loss: using the zebrafish lateral line to screen for drugs that prevent and cause hearing loss. Drug Discov. Today 15, 265-271.

Ou, H. C., Keating, S., Wu, P., Simon, J. A., Raible, D. W., Rubel, E. W., 2012. Quinoline Ring Derivatives Protect Against

Aminoglycoside-Induced Hair Cell Death in the Zebrafish Lateral Line. J Assoc Res Otolaryngol.
Owens, K. N., Cunningham, D. E., MacDonald, G., Rubel, E. W., Raible, D. W., Pujol, R., 2007. Ultrastructural analysis of aminoglycoside-induced hair cell death in the zebrafish lateral line reveals an early mitochondrial response. J. Comp. Neurol. 502, 522-543.
Owens, K. N., Santos, F., Roberts, B., Linbo, T., Coffin, A. B., Knisely, A. J., Simon, J. A., Rubel, E. W., Raible, D. W., 2008. Identification of genetic and chemical modulators of zebrafish mechanosensory hair cell death. PLoS Genet. 4, e1000020.
Owens, K. N., Coffin, A. B., Hong, L. S., Bennett, K. O., Rubel, E. W., Raible, D. W., 2009. Response of mechanosensory hair cells of the zebrafish lateral line to aminoglycosides reveals distinct cell death pathways. Hear. Res. 253, 32-41.
Paffenholz, R., Bergstrom, R. A., Pasutto, F., Wabnitz, P., Munroe, R. J., Jagla, W., Heinzmann, U., Marquardt, A., Bareiss, A., Laufs, J., Russ, A., Stumm, G., Schimenti, J. C., Bergstrom, D. E., 2004. Vestibular defects in head-tilt mice result from mutations in Nox3, encoding an NADPH oxidase. Genes Dev 18, 486-491.
Pardo-Martin, C., Chang, T. Y., Koo, B. K., Gilleland, C. L., Wasserman, S. C., Yanik, M. F., 2010. High-throughput in vivo vertebrate screening. Nat Methods 7, 634-636.
Parinov, S., Kondrichin, I., Korzh, V., Emelyanov, A., 2004. Tol2 transposon-mediated enhancer trap to identify developmentally regulated zebrafish genes in vivo. Developmental Dynamics 231, 449-459.
Park, S. J., Park, S. H., Chang, J. W., Choi, J., Jung, H. H., Im, G. J., 2012. Protective effect of klotho protein against cisplatin ototoxicity in an auditory cell line. Journal of Laryngology and Otology, 1-7.
Partridge, B. L., Pitcher, T. J., 1980. The Sensory Basis of Fish Schools - Relative Roles of Lateral Line and Vision. Journal of Comparative Physiology 135, 315-325.
Peng, B. G., Chen, S., Lin, X., 2003. Aspirin selectively augmented N-methyl-D-aspartate types of glutamate responses in cultured spiral ganglion neurons of mice. Neurosci Lett 343, 21-24.
Peters, U., Preisler-Adams, S., Hebeisen, A., Hahn, M., Seifert, E., Lanvers, C., Heinecke, A., Horst, J., Jurgens, H., Lamprecht-Dinnesen, A., 2000. Glutathione S-transferase genetic polymorphisms and individual sensitivity to the ototoxic effect of cisplatin. Anticancer Drugs 11, 639-643.

Peterson, A. A., Hancock, R. E., McGroarty, E. J., 1985. Binding of polycationic antibiotics and polyamines to lipopolysaccharides of Pseudomonas aeruginosa. J Bacteriol 164, 1256-1261.

Pfannenstiel, S. C., Praetorius, M., Plinkert, P. K., Brough, D. E., Staecker, H., 2009. Bcl-2 Gene Therapy Prevents Aminoglycoside-Induced Degeneration of Auditory and Vestibular Hair Cells. Audiology and Neuro-Otology 14, 254-266.

Pirvola, U., Xing-Qun, L., Virkkala, J., Saarma, M., Murakata, C., Camoratto, A. M., Walton, K. M., Ylikoski, J., 2000. Rescue of hearing, auditory hair cells, and neurons by CEP-1347/KT7515, an inhibitor of c-Jun N-terminal kinase activation. J Neurosci 20, 43-50.

Prajsnar, T. K., Cunliffe, V. T., Foster, S. J., Renshaw, S. A., 2008. A novel vertebrate model of Staphylococcus aureus infection reveals phagocyte-dependent resistance of zebrafish to non-host specialized pathogens. Cell Microbiol 10, 2312-2325.

Prepageran, N., Scott, A. R., Rutka, J. 2004. Ototoxicity of Loop Diuretics. Ototoxicity. Roland, C,Rutka, J. New York, BC Decker**:** 43-49.

Prepageran, N., Rutka, J. 2004. Salicylates, Nonsteroidal Anti-inflammatory Drugs, Quinine, and Heavy Metals. Ototoxicity. Roland, P,Rutka, J. New York, BC Decker**:** 28-41.

Prezant, T. R., Agapian, J. V., Bohlman, M. C., Bu, X., Oztas, S., Qiu, W. Q., Arnos, K. S., Cortopassi, G. A., Jaber, L., Rotter, J. I., et al., 1993. Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndromic deafness. Nat Genet 4, 289-294.

Priuska, E. M., Schacht, J., 1995. Formation of free radicals by gentamicin and iron and evidence for an iron gentamicin complex. Biochemical Pharmacology 50, 1749-1752.

Priuska, E. M., Clark-Baldwin, K., Pecoraro, V. L., Schacht, J., 1998. NMR studies of iron-gentamicin complexes and the implications for aminoglycoside toxicity. Inorganica Chimica Acta 273, 85-91.

Puel, J. L., Guitton, M. J., 2007. Salicylate-induced tinnitus: molecular mechanisms and modulation by anxiety. Prog Brain Res 166, 141-146.

Raible, D. W., Kruse, G. J., 2000. Organization of the lateral line system in embryonic zebrafish. J. Comp. Neurol. 421, 189-198.

Ramcharitar, J., Selckmann, G. M., 2010. Differential ablation of sensory receptors underlies ototoxin-induced shifts in auditory

thresholds of the goldfish (Carassius auratus). J. Appl. Toxicol. 30, 536-541.
Ramcharitar, J. U., Brack, C. L., 2010. Physiological dimensions of ototoxic responses in a model fish species. J. Clin. Neurosci. 17, 103-106.
Rankin, C. H., Abrams, T., Barry, R. J., Bhatnagar, S., Clayton, D. F., Colombo, J., Coppola, G., Geyer, M. A., Glanzman, D. L., Marsland, S., McSweeney, F. K., Wilson, D. A., Wu, C. F., Thompson, R. F., 2009. Habituation revisited: an updated and revised description of the behavioral characteristics of habituation. Neurobiol. Learn. Mem. 92, 135-138.
Redfern, W. S., Wakefield, I. D., Prior, H., Pollard, C. E., Hammond, T. G., Valentin, J. P., 2002. Safety pharmacology--a progressive approach. Fundam Clin Pharmacol 16, 161-173.
Redfern, W. S., Waldron, G., Winter, M. J., Butler, P., Holbrook, M., Wallis, R., Valentin, J. P., 2008. Zebrafish assays as early safety pharmacology screens: paradigm shift or red herring? J. Pharmacol. Toxicol. Methods 58, 110-117.
Renshaw, S. A., Loynes, C. A., Trushell, D. M., Elworthy, S., Ingham, P. W., Whyte, M. K., 2006. A transgenic zebrafish model of neutrophilic inflammation. Blood 108, 3976-3978.
Richards, F. M., Alderton, W. K., Kimber, G. M., Liu, Z., Strang, I., Redfern, W. S., Valentin, J. P., Winter, M. J., Hutchinson, T. H., 2008. Validation of the use of zebrafish larvae in visual safety assessment. J Pharmacol Toxicol Methods 58, 50-58.
Richardson, G. P., Russell, I. J., 1991. Cochlear Cultures as a Model System for Studying Aminoglycoside Induced Ototoxicity. Hearing Research 53, 293-311.
Richardson, G. P., Forge, A., Kros, C. J., Fleming, J., Brown, S. D., Steel, K. P., 1997. Myosin VIIA is required for aminoglycoside accumulation in cochlear hair cells. J Neurosci 17, 9506-9519.
Richendrfer, H., Pelkowski, S. D., Colwill, R. M., Creton, R., 2012. On the edge: pharmacological evidence for anxiety-related behavior in zebrafish larvae. Behav Brain Res 228, 99-106.
Ridges, S., Heaton, W. L., Joshi, D., Choi, H., Eiring, A., Batchelor, L., Choudhry, P., Manos, E. J., Sofla, H., Sanati, A., Welborn, S., Agarwal, A., Spangrude, G. J., Miles, R. R., Cox, J. E., Frazer, J. K., Deininger, M., Balan, K., Sigman, M., Muschen, M., Perova, T., Johnson, R., Montpellier, B., Guidos, C. J., Jones, D. A., Trede, N. S., 2012. Zebrafish screen identifies novel compound with selective toxicity against leukemia. Blood 119, 5621-5631.

Riedemann, L., Lanvers, C., Deuster, D., Peters, U., Boos, J., Jurgens, H., am Zehnhoff-Dinnesen, A., 2008. Megalin genetic polymorphisms and individual sensitivity to the ototoxic effect of cisplatin. Pharmacogenomics J 8, 23-28.

Riga, M. G., Chelis, L., Kakolyris, S., Papadopoulos, S., Stathakidou, S., Chamalidou, E., Xenidis, N., Amarantidis, K., Dimopoulos, P., Danielides, V., 2011. Transtympanic Injections of N-acetylcysteine for the Prevention of Cisplatin-induced Ototoxicity: A Feasible Method With Promising Efficacy. Am J Clin Oncol.

Rihel, J., Prober, D. A., Arvanites, A., Lam, K., Zimmerman, S., Jang, S., Haggarty, S. J., Kokel, D., Rubin, L. L., Peterson, R. T., Schier, A. F., 2010. Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. Science 327, 348-351.

Rihel, J., Schier, A. F., 2012. Behavioral screening for neuroactive drugs in zebrafish. Dev Neurobiol 72, 373-385.

Rivolta, M. N., Grix, N., Lawlor, P., Ashmore, J. F., Jagger, D. J., Holley, M. C., 1998. Auditory hair cell precursors immortalized from the mammalian inner ear. Proc Biol Sci 265, 1595-1603.

Rivolta, M. N., Holley, M. C., 2002. Cell lines in inner ear research. J Neurobiol 53, 306-318.

RNID, 2007. Opportunity Blocked: The employment experiences of deaf and hard of hearing people.

Roberts, A. C., Reichl, J., Song, M. Y., Dearinger, A. D., Moridzadeh, N., Lu, E. D., Pearce, K., Esdin, J., Glanzman, D. L., 2011. Habituation of the C-Start Response in Larval Zebrafish Exhibits Several Distinct Phases and Sensitivity to NMDA Receptor Blockade. PLoS One 6.

Rosenberg, B., Vancamp, L., Krigas, T., 1965. Inhibition of Cell Division in Escherichia Coli by Electrolysis Products from a Platinum Electrode. Nature 205, 698-699.

Rosenberg, B., VanCamp, L., Trosko, J. E., Mansour, V. H., 1969. Platinum compounds: a new class of potent antitumour agents. Nature 222, 385-386.

Rosenberg, B., 1985. Fundamental-Studies with Cisplatin. Cancer 55, 2303-2316.

Ross, C. J., Katzov-Eckert, H., Dube, M. P., Brooks, B., Rassekh, S. R., Barhdadi, A., Feroz-Zada, Y., Visscher, H., Brown, A. M., Rieder, M. J., Rogers, P. C., Phillips, M. S., Carleton, B. C., Hayden, M. R., 2009. Genetic variants in TPMT and COMT are associated with hearing loss in children receiving cisplatin chemotherapy. Nat Genet 41, 1345-1349.

Ross, W. E., Ewig, R. A., Kohn, K. W., 1978. Differences between melphalan and nitrogen mustard in the formation and removal of DNA cross-links. Cancer Res 38, 1502-1506.
Rotstein, C., Mandell, L. 2004. Clinical Aminoglycoside Toxicity. Ototoxicity. Roland, P,Rutka, J. New York, BC Decker**:** 82-92.
Rutman, D., 1989. The impact and experience of adventitious deafness. Am Ann Deaf 134, 305-311.
Ruttiger, L., Ciuffani, J., Zenner, H. P., Knipper, M., 2003. A behavioral paradigm to judge acute sodium salicylate-induced sound experience in rats: a new approach for an animal model on tinnitus. Hear Res 180, 39-50.
Rybak, L. P., 1988. Ototoxicity of ethacrynic acid (a persistent clinical problem). Journal of Laryngology and Otology 102, 518-520.
Rybak, L. P., 1993. Ototoxicity of loop diuretics. Otolaryngol Clin North Am 26, 829-844.
Rybak, L. P., Whitworth, C. A., Mukherjea, D., Ramkumar, V., 2007. Mechanisms of cisplatin-induced ototoxicity and prevention. Hear. Res. 226, 157-167.
Rybak, L. P., Ramkumar, V., 2007. Ototoxicity. Kidney Int. 72, 931-935.
Rybak, L. P., Mukherjea, D., Jajoo, S., Ramkumar, V., 2009. Cisplatin ototoxicity and protection: clinical and experimental studies. Tohoku J Exp Med 219, 177-186.
Sanger. from http://www.sanger.ac.uk/Projects/D_rerio/.
Santos, F., MacDonald, G., Rubel, E. W., Raible, D. W., 2006. Lateral line hair cell maturation is a determinant of aminoglycoside susceptibility in zebrafish (Danio rerio). Hear. Res. 213, 25-33.
Sarrazin, A. F., Villablanca, E. J., Nunez, V. A., Sandoval, P. C., Ghysen, A., Allende, M. L., 2006. Proneural gene requirement for hair cell differentiation in the zebrafish lateral line. Dev Biol 295, 534-545.
Schacht, J. 2004. Mechanisms for Aminoglycoside Ototoxicity: Basic Science Research. Ototoxicity. Roland, P,Rutka, J. New York, BC Decker**:** 93-100.
Schaefer, S. D., Post, J. D., Close, L. G., Wright, C. G., 1985. Ototoxicity of low- and moderate-dose cisplatin. Cancer 56, 1934-1939.
Schatz, A., Bugie, E., Waksman, S. A., 2005. Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. (original publication from1944). Clin Orthop Relat Res, 3-6.

Scheunemann, A. E., Graham, W. D., Vendeix, F. A., Agris, P. F., 2010. Binding of aminoglycoside antibiotics to helix 69 of 23S rRNA. Nucleic Acids Res 38, 3094-3105.
Schibler, A., Malicki, J., 2007. A screen for genetic defects of the zebrafish ear. Mechanisms of Development 124, 592-604.
Schmitt, N. C., Rubel, E. W., Nathanson, N. M., 2009. Cisplatin-induced hair cell death requires STAT1 and is attenuated by epigallocatechin gallate. J Neurosci 29, 3843-3851.
Schnorr, S. J., Steenbergen, P. J., Richardson, M. K., Champagne, D. L., 2012. Measuring thigmotaxis in larval zebrafish. Behav Brain Res 228, 367-374.
Schwartz, G. H., David, D. S., Riggio, R. R., Stenzel, K. H., Rubin, A. L., 1970. Ototoxicity induced by furosemide. N Engl J Med 282, 1413-1414.
Scott, P. M., Griffiths, M. V., 1994. A clinical review of ototoxicity. Clin Otolaryngol Allied Sci 19, 3-8.
Seiler, C., Nicolson, T., 1999. Defective calmodulin-dependent rapid apical endocytosis in zebrafish sensory hair cell mutants. J Neurobiol 41, 424-434.
Sha, S. H., Schacht, J., 1999a. Formation of reactive oxygen species following bioactivation of gentamicin. Free Radic Biol Med 26, 341-347.
Sha, S. H., Schacht, J., 1999b. Stimulation of free radical formation by aminoglycoside antibiotics. Hear Res 128, 112-118.
Sha, S. H., Taylor, R., Forge, A., Schacht, J., 2001a. Differential vulnerability of basal and apical hair cells is based on intrinsic susceptibility to free radicals. Hear Res 155, 1-8.
Sha, S. H., Zajic, G., Epstein, C. J., Schacht, J., 2001b. Overexpression of copper/zinc-superoxide dismutase protects from kanamycin-induced hearing loss. Audiol Neurootol 6, 117-123.
Shield, B. 2006. Evaluation of the Social and Economic Costs of Hearing Impairment, A report for Hear-It.
Sillar, K. T., 2009. Escape behaviour: reciprocal inhibition ensures effective escape trajectory. Curr Biol 19, R697-699.
Sison, M., Gerlai, R., 2011. Behavioral performance altering effects of MK-801 in zebrafish (Danio rerio). Behav Brain Res 220, 331-337.
Slattery, E. L., Warchol, M. E., 2010. Cisplatin ototoxicity blocks sensory regeneration in the avian inner ear. J Neurosci 30, 3473-3481.
Sly, D. J., Hampson, A. J., Minter, R. L., Heffer, L. F., Li, J., Millard, R. E., Winata, L., Niasari, A., O'Leary, S. J., 2012. Brain-

derived neurotrophic factor modulates auditory function in the hearing cochlea. J Assoc Res Otolaryngol 13, 1-16.
Sneader, W., 2000. The discovery of aspirin: a reappraisal. BMJ 321, 1591-1594.
So, H., Kim, H., Lee, J. H., Park, C., Kim, Y., Kim, E., Kim, J. K., Yun, K. J., Lee, K. M., Lee, H. Y., Moon, S. K., Lim, D. J., Park, R., 2007. Cisplatin cytotoxicity of auditory cells requires secretions of proinflammatory cytokines via activation of ERK and NF-kappaB. J Assoc Res Otolaryngol 8, 338-355.
So, H., Kim, H., Kim, Y., Kim, E., Pae, H. O., Chung, H. T., Kim, H. J., Kwon, K. B., Lee, K. M., Lee, H. Y., Moon, S. K., Park, R., 2008. Evidence that cisplatin-induced auditory damage is attenuated by downregulation of pro-inflammatory cytokines via Nrf2/HO-1. Jaro-Journal of the Association for Research in Otolaryngology 9, 290-306.
Song, B. B., Schacht, J., 1996. Variable efficacy of radical scavengers and iron chelators to attenuate gentamicin ototoxicity in guinea pig in vivo. Hear Res 94, 87-93.
Song, B. B., Anderson, D. J., Schacht, J., 1997. Protection from gentamicin ototoxicity by iron chelators in guinea pig in vivo. J Pharmacol Exp Ther 282, 369-377.
Spence, R., Gerlach, G., Lawrence, C., Smith, C., 2008. The behaviour and ecology of the zebrafish, Danio rerio. Biol Rev Camb Philos Soc 83, 13-34.
Stevens, J. L., Baker, T. K., 2009. The future of drug safety testing: expanding the view and narrowing the focus. Drug Discov Today 14, 162-167.
Sturgeon, J. 2004. Clinical Uses of Cisplatin. Ototoxicity. Roland, P,Rutka, J. New York, BC Decker**:** 51-57.
Sugahara, K., Rubel, E. W., Cunningham, L. L., 2006. JNK signaling in neomycin-induced vestibular hair cell death. Hear Res 221, 128-135.
Suli, A., Watson, G. M., Rubel, E. W., Raible, D. W., 2012. Rheotaxis in larval zebrafish is mediated by lateral line mechanosensory hair cells. PLoS One 7, e29727.
Tabuchi, K., Pak, K., Chavez, E., Ryan, A. F., 2007. Role of inhibitor of apoptosis protein in gentamicin-induced cochlear hair cell damage. Neuroscience 149, 213-222.
Takumida, M., Anniko, M., 2002. Simultaneous detection of both nitric oxide and reactive oxygen species in guinea pig vestibular sensory cells. ORL J Otorhinolaryngol Relat Spec 64, 143-147.

Tange, R. A., Dreschler, W. A., Claessen, F. A., Perenboom, R. M., 1997. Ototoxic reactions of quinine in healthy persons and patients with Plasmodium falciparum infection. Auris Nasus Larynx 24, 131-136.

Taylor, R. R., Nevill, G., Forge, A., 2008. Rapid hair cell loss: a mouse model for cochlear lesions. J Assoc Res Otolaryngol 9, 44-64.

Thomas, A., Herbst, K. G., 1980. Social and psychological implications of acquired deafness for adults of employment age. Br J Audiol 14, 76-85.

Thompson, R. F., Spencer, W. A., 1966. Habituation: a model phenomenon for the study of neuronal substrates of behavior. Psychol. Rev. 73, 16-43.

Tingaud-Sequeira, A., Ouadah, N., Babin, P. J., 2011. Zebrafish obesogenic test: a tool for screening molecules that target adiposity. Journal of Lipid Research 52, 1765-1772.

Ton, C., Parng, C., 2005. The use of zebrafish for assessing ototoxic and otoprotective agents. Hear. Res. 208, 79-88.

Tunstall, M. J., Gale, J. E., Ashmore, J. F., 1995. Action of salicylate on membrane capacitance of outer hair cells from the guinea-pig cochlea. J Physiol 485 ( Pt 3), 739-752.

Tuper, G., Ahmad, N., Seidman, M., 2005. Mechanisms of Ototoxicity. Perspectives on Hearing and Hearing Disorders: Research and Diagnostics (American Speech-Language-Hearing Association), 2-10.

Valentin, G., Haas, P., Gilmour, D., 2007. The chemokine SDF1a coordinates tissue migration through the spatially restricted activation of Cxcr7 and Cxcr4b. Curr Biol 17, 1026-1031.

Valentin, J. P., Hammond, T., 2008. Safety and secondary pharmacology: successes, threats, challenges and opportunities. J Pharmacol Toxicol Methods 58, 77-87.

van Ham, T. J., Mapes, J., Kokel, D., Peterson, R. T., 2010. Live imaging of apoptotic cells in zebrafish. Faseb Journal 24, 4336-4342.

Van Trump, W. J., Coombs, S., Duncan, K., McHenry, M. J., 2010. Gentamicin is ototoxic to all hair cells in the fish lateral line system. Hear. Res. 261, 42-50.

Vane, J. R., 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat New Biol 231, 232-235.

Vane, J. R., Botting, R. M., 2003. The mechanism of action of aspirin. Thromb Res 110, 255-258.

VEDA, 2010. Vestibular Disorders: An Overview.

Vio, M. M., Holme, R. H., 2005. Hearing loss and tinnitus: 250 million people and a US$10 billion potential market. Drug Discov Today 10, 1263-1265.
Vlasits, A. L., Simon, J. A., Raible, D. W., Rubel, E. W., Owens, K. N., 2012. Screen of FDA-approved drug library reveals compounds that protect hair cells from aminoglycosides and cisplatin. Hear Res.
Vogt, A., Cholewinski, A., Shen, X., Nelson, S. G., Lazo, J. S., Tsang, M., Hukriede, N. A., 2009. Automated image-based phenotypic analysis in zebrafish embryos. Dev Dyn 238, 656-663.
Vogt, A., Codore, H., Day, B. W., Hukriede, N. A., Tsang, M., 2010. Development of automated imaging and analysis for zebrafish chemical screens. J Vis Exp.
Voldrich, L., 1965. The kinetics of streptomycin, kanamycin and neomycin in the inner ear. Acta Otolaryngol 60, 243-248.
Waissbluth, S., Salehi, P., He, X., Daniel, S. J., 2012. Systemic dexamethasone for the prevention of cisplatin-induced ototoxicity. Eur Arch Otorhinolaryngol.
Waksman, S. A., Lechevalier, H. A., 1949. Neomycin, a New Antibiotic Active against Streptomycin-Resistant Bacteria, including Tuberculosis Organisms. Science 109, 305-307.
Walker, S. L., Ariga, J., Mathias, J. R., Coothankandaswamy, V., Xie, X., Distel, M., Koster, R. W., Parsons, M. J., Bhalla, K. N., Saxena, M. T., Mumm, J. S., 2012. Automated reporter quantification in vivo: high-throughput screening method for reporter-based assays in zebrafish. PLoS One 7, e29916.
Wang, B. M., Weiner, N. D., Takada, A., Schacht, J., 1984. Characterization of aminoglycoside-lipid interactions and development of a refined model for ototoxicity testing. Biochem Pharmacol 33, 3257-3262.
Wang, J., Lloyd Faulconbridge, R. V., Fetoni, A., Guitton, M. J., Pujol, R., Puel, J. L., 2003a. Local application of sodium thiosulfate prevents cisplatin-induced hearing loss in the guinea pig. Neuropharmacology 45, 380-393.
Wang, J., Van de Water, T. R., Bonny, C., de Ribaupierre, F., Puel, J. L., Zine, A., 2003b. A peptide inhibitor of c-Jun N-terminal kinase protects against both aminoglycoside and acoustic trauma-induced auditory hair cell death and hearing loss. Journal of Neuroscience 23, 8596-8607.
Wang, J., Ladrech, S., Pujol, R., Brabet, P., Van De Water, T. R., Puel, J. L., 2004. Caspase inhibitors, but not c-Jun NH2-

terminal kinase inhibitor treatment, prevent cisplatin-induced hearing loss. Cancer Res 64, 9217-9224.
Wang, Q., Steyger, P. S., 2009. Trafficking of Systemic Fluorescent Gentamicin into the Cochlea and Hair Cells. Jaro-Journal of the Association for Research in Otolaryngology 10, 205-219.
Waterman, R. E., Bell, D. H., 1984. Epithelial fusion during early semicircular canal formation in the embryonic zebrafish, Brachydanio rerio. Anat Rec 210, 101-114.
Weatherly, R. A., Owens, J. J., Catlin, F. I., Mahoney, D. H., 1991. cis-platinum ototoxicity in children. Laryngoscope 101, 917-924.
Weber, D. N., 2006. Dose-dependent effects of developmental mercury exposure on C-start escape responses of larval zebrafish Danio rerio. J. Fish Biol. 69, 75-94.
Weber, T., Gopfert, M. C., Winter, H., Zimmermann, U., Kohler, H., Meier, A., Hendrich, O., Rohbock, K., Robert, D., Knipper, M., 2003. Expression of prestin-homologous solute carrier (SLC26) in auditory organs of nonmammalian vertebrates and insects. Proc Natl Acad Sci U S A 100, 7690-7695.
Wei, X., Zhao, L., Liu, J., Dodel, R. C., Farlow, M. R., Du, Y., 2005. Minocycline prevents gentamicin-induced ototoxicity by inhibiting p38 MAP kinase phosphorylation and caspase 3 activation. Neuroscience 131, 513-521.
Weinstein, M. J., Luedemann, G. M., Oden, E. M., Wagman, G. H., 1963. Gentamicin, a New Broad-Spectrum Antibiotic Complex. Antimicrob Agents Chemother (Bethesda) 161, 1-7.
Weiss, S. A., Zottoli, S. J., Do, S. C., Faber, D. S., Preuss, T., 2006. Correlation of C-start behaviors with neural activity recorded from the hindbrain in free-swimming goldfish (Carassius auratus). J Exp Biol 209, 4788-4801.
Whitfield, T. T., Granato, M., van Eeden, F. J., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C. P., Jiang, Y. J., Kane, D. A., Kelsh, R. N., Mullins, M. C., Odenthal, J., Nusslein-Volhard, C., 1996. Mutations affecting development of the zebrafish inner ear and lateral line. Development 123, 241-254.
Whitfield, T. T., Riley, B. B., Chiang, M. Y., Phillips, B., 2002. Development of the zebrafish inner ear. Dev. Dyn. 223, 427-458.
WHO, 2012. Deafness and hearing impairment factsheet no. 300.
Wilkins, D. E., Hallett, M., Wess, M. M., 1986. Audiogenic Startle Reflex of Man and Its Relationship to Startle Syndromes - a Review. Brain 109, 561-573.

Williams, J. A., Holder, N., 2000. Cell turnover in neuromasts of zebrafish larvae. Hear Res 143, 171-181.
Williams, S. E., Zenner, H. P., Schacht, J., 1987. Three molecular steps of aminoglycoside ototoxicity demonstrated in outer hair cells. Hear Res 30, 11-18.
Willott, J. F., Turner, J. G., Carlson, S., Ding, D. L., Bross, L. S., Falls, W. A., 1998. The BALB/c mouse as an animal model for progressive sensorineural hearing loss. Hearing Research 115, 162-174.
Wimmer, C., Mees, K., Stumpf, P., Welsch, U., Reichel, O., Suckfull, M., 2004. Round window application of D-methionine, sodium thiosulfate, brain-derived neurotrophic factor, and fibroblast growth factor-2 in cisplatin-induced ototoxicity. Otol Neurotol 25, 33-40.
Winter, M. J., Redfern, W. S., Hayfield, A. J., Owen, S. F., Valentin, J. P., Hutchinson, T. H., 2008. Validation of a larval zebrafish locomotor assay for assessing the seizure liability of early-stage development drugs. J. Pharmacol. Toxicol. Methods 57, 176-187.
Wolman, M. A., Jain, R. A., Liss, L., Granato, M., 2011. Chemical modulation of memory formation in larval zebrafish. Proc Natl Acad Sci U S A 108, 15468-15473.
Woodcock, J., Moazed, D., Cannon, M., Davies, J., Noller, H. F., 1991. Interaction of antibiotics with A- and P-site-specific bases in 16S ribosomal RNA. EMBO J 10, 3099-3103.
Wright, C. G., Schaefer, S. D., 1982. Inner ear histopathology in patients treated with cis-platinum. Laryngoscope 92, 1408-1413.
Xiao, T., Roeser, T., Staub, W., Baier, H., 2005. A GFP-based genetic screen reveals mutations that disrupt the architecture of the zebrafish retinotectal projection. Development 132, 2955-2967.
Xie, J., Talaska, A. E., Schacht, J., 2011. New developments in aminoglycoside therapy and ototoxicity. Hear Res 281, 28-37.
Yancey, A., Harris, M. S., Egbelakin, A., Gilbert, J., Pisoni, D. B., Renbarger, J., 2012. Risk factors for cisplatin-associated ototoxicity in pediatric oncology patients. Pediatr Blood Cancer 59, 144-148.
Yang, T. H., Liu, S. H., Young, Y. H., 2010. A novel inner ear monitoring system for evaluating ototoxicity of gentamicin eardrops in guinea pigs. Laryngoscope 120, 1220-1226.
Ylikoski, J., Xing-Qun, L., Virkkala, J., Pirvola, U., 2002. Blockade of c-Jun N-terminal kinase pathway attenuates gentamicin-

induced cochlear and vestibular hair cell death. Hear Res 166, 33-43.
Yonezawa, A., Inui, K., 2011. Organic cation transporter OCT/SLC22A and H(+)/organic cation antiporter MATE/SLC47A are key molecules for nephrotoxicity of platinum agents. Biochem Pharmacol 81, 563-568.
Yorgason, J. G., Luxford, W., Kalinec, F., 2011. In vitro and in vivo models of drug ototoxicity: studying the mechanisms of a clinical problem. Expert Opin Drug Metab Toxicol 7, 1521-1534.
Zeddies, D. G., Fay, R. R., 2005. Development of the acoustically evoked behavioral response in zebrafish to pure tones. J. Exp. Biol. 208, 1363-1372.
Zhang, M., Liu, W., Ding, D., Salvi, R., 2003. Pifithrin-alpha suppresses p53 and protects cochlear and vestibular hair cells from cisplatin-induced apoptosis. Neuroscience 120, 191-205.
Zottoli, S. J., 1977. Correlation of the startle reflex and Mauthner cell auditory responses in unrestrained goldfish. J Exp Biol 66, 243-254.

# Appendix 1 Macro instructions

**Table A1.1:** Macro design for "Startle template FULL VERSION" worksheet.

| Macro | Command | Brief description of task performed | Intermediate/manual step |
|---|---|---|---|
| 1 | Ctrl + i | Cleans up raw data: copies data into new sheet *copy of raw data*, sorts by *an,* deletes *an* values equal to 1, removes unwanted columns, copies data into *cleaned up data.* Inserts columns *plate number* and *stimulus*. | |
| 2 | Ctrl + j | Assigns plate reference number, assigns stimulus reference, alters *animal* column so that animals from plate 2 = *.2*. Sorts data ready for removal of inactive larvae (*removal of undetected larvae*). | Manually delete undetected larvae |
| 3 | Ctrl + k | Splits data into individual plates, copies each dataset into *summary for stimuli* sheets, deletes unwanted columns. Copies data from *summary for stimuli* sheets into *find stim time* sheets and orders by *stimulus frequency* then *start time*. | Manually remove outliers (marked *reject*) by highlighting then deleting. Manually copy data from identified startle time (e.g.4-5 s) into *startle vs. conc* sheet, for each stimulus. Sort by *stimulus* then *concentration/dose*. |
| 4 | Ctrl + l | Moves summary data for each stimulus into data table for all three plates. Orders for each frequency and makes graphs. | |
| 5 | Ctrl + m | Makes summary data for all concentrations and frequencies | |
| 6 | Ctrl + n | Provides raw data for startle. | Delete the unnecessary sheets (edited version only). |
| 7 | Ctrl + o | Produces data which can be used to analyse individual trials (A, B, C) or all three trials (A-C) using statistical methods. | Copy data from *raw summary all 3* into *all/raw data per fish*. Sort data by dose, then animal (custom list), then stimulus frequency. Alter N to 0 if there is no data for the larva and delete DIV#. Use this for summary data for all of the trials. |

**Table A1.2:** Macro design for "summary of all trials" worksheet.

| Macro number | Command | Brief description of task performed | Intermediate/manual step |
|---|---|---|---|
| | | | For each trial (A, B, C) copy and paste data from *final raw per Hz* |
| 1 | Ctrl + q | Summarises trials A, B and C. | |
| 2 | Ctrl + r | Copies and pastes *lar. dist*. values for each frequency at each individual dose. | Copy and paste data directly from *all data per fish* into *all freq all 3 rep*. |
| 3 | Ctrl +shift+ q | Provides all data over three trials, combining frequency data | |
| 4 | Ctrl +shift+ r | Copies over average large distance data per fish, for statistical analysis. | Copy into Graphpad for analysis. |

# Appendix 2 Functional assays (proforma)

**TEST COMPOUND INFORMATION**

| DATE WEIGHED | COMPOUND | WEIGHT AND BALANCE REF. | VOLUME LIQUID REQUIRED FOR STOCK | STOCK CONCENTRATION/ DATE STOCK MADE | DILTUION NEEDED | pH TOP CONCENTR -ATION | pH METER USED |
|---|---|---|---|---|---|---|---|
| | | | | | | | |

SOLVENT/WATER CONTROL USED: …….

FINAL pH of SOLVENT/WATER CONTROL: ……..

FINAL % SOLVENT IN S/C: ……

DATE MADE: …….

**LIGHT ADJUSTMENT PHASE/PLATE TIMES**

PLATE 1: ……..arrayed from a mix of …….plates

PLATE 2: …….arrayed from a mix of……plates

PLATE 3: ……arrayed from a mix of …....plates

Pipettes used: …….. (1mL 1 X E3 clear)

**RHEOTAXIS SCORES BEFORE TREATMENT:**

PLATE 1: (tick)

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **A** | | | | | | |
| **B** | | | | | | |
| **C** | | | | | | |
| **D** | | | | | | |

**REHEOTAXIS SCORES BEFORE TREATMENT:**

PLATE 2: (tick)

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **A** | | | | | | |
| **B** | | | | | | |
| **C** | | | | | | |
| **D** | | | | | | |

PLATE 3: (tick)

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **A** | | | | | | |
| **B** | | | | | | |
| **C** | | | | | | |
| **D** | | | | | | |

**MTC SCORES BEFORE TREATMENT**

Plate 1: (score)

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **A** | | | | | | |
| **B** | | | | | | |
| **C** | | | | | | |
| **D** | | | | | | |

Plate 2: (score)

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **A** | | | | | | |
| **B** | | | | | | |
| **C** | | | | | | |
| **D** | | | | | | |

**MTC SCORES BEFORE TREATMENT:**

Plate 3: (score)

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **A** | | | | | | |
| **B** | | | | | | |
| **C** | | | | | | |
| **D** | | | | | | |

**PLATE CONFIGURATION AND TREATMENT INFORMATION (BASED ON RANDOM NUMBER GENERATION):**

PLATE 1:

Room Temp at time of treatment: …….

| | Positive control | S/C | MTC (HC) | EC50 (HC) | S/C | S/C keep |
|---|---|---|---|---|---|---|
| **A** | | | | | | |
| **B** | | | | | | |
| **C** | | | | | | |
| **D** | | | | | | |

TIME OF TREATMENT: …….

PIPETTES USED: ……

TIME OF WASHES (3X) IN 1X E3 (clear/no methylene blue): …….

PLATE 2:

Room Temp at time of treatment: …….

| | EC50 (HC) | S/C | MTC (HC) | Positive Control | S/C | S/C Keep |
|---|---|---|---|---|---|---|
| **A** | | | | | | |
| **B** | | | | | | |
| **C** | | | | | | |
| **D** | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | |

TIME OF TREATMENT: …….

PIPETTES USED: ……

TIME OF WASHES (3X) IN 1X E3 (clear/no methylene blue): …….

**PLATE CONFIGURATION AND TREATMENT INFORMATION (BASED ON RANDOM NUMBER GENERATION):**

PLATE 3:

Room temp at time of treatment: ……..

| | EC50 (HC) | Positive control | S/C (Keep) | S/C | MTC (HC) | S/C |
|---|---|---|---|---|---|---|
| **A** | | | | | | |
| **B** | | | | | | |
| **C** | | | | | | |
| **D** | | | | | | |

TIME OF TREATMENT: ……..

PIPETTES USED: ……

TIME OF WASHES (3X) IN 1X E3 (clear/no methylene blue): …….

**PLATE 1 PARADIGM:**

Plate 1 placed into startle arena at: ……..

Baseline recording taken for 60s at: …… (30fps)

Room Temp at start: ……. Room Temp at end: ……..

**OBSERVATIONS** (over 10 minutes from……..until…….……):

(I=inactive, H = hyperactive, Si = Lying on side, O = other orientation issue/vestibular)

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **A** | | | | | | |
| **B** | | | | | | |
| **C** | | | | | | |
| **D** | | | | | | |

**HEARING TEST PARADIGM:**

START TIME: ……

40 Hz (540 milliseconds, 2.3 Vpp, -15dB)…… (tick)

50 Hz (440 milliseconds, 1.6 Vpp, -15dB)……. (tick)

100 Hz (440 milliseconds, 2.1 Vpp, -25dB)…… (tick)

150 Hz (440 milliseconds, 2.7 Vpp, -35dB)……... (tick)

200 Hz (440 milliseconds, 2.2 Vpp, -30dB)…… (tick)

300 Hz (440 milliseconds, 3.8 Vpp, -40dB)…… (tick)

400 Hz (440 milliseconds, 4.3 Vpp, -41.25dB)…….. (tick)

500 Hz (440 milliseconds, 4.5 Vpp, -30dB)…… (tick)

END TIME: ……..

SAVED AS FOLDER:……

**RHEOTAXIS AFTER TREATMENT:** (tick)

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **A** | | | | | | |
| **B** | | | | | | |
| **C** | | | | | | |
| **D** | | | | | | |

**MTC AFTER TREATMENT:**

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **A** | | | | | | |
| **B** | | | | | | |
| **C** | | | | | | |
| **D** | | | | | | |

**PLATE 2 PARADIGM:**

Plate 2 placed into startle arena at: ……..

Baseline recording taken for ……. at: …… (30 fps)

Room Temp at start: ……. Room Temp at end: ……..

**OBSERVATIONS** (over 10 minutes from……..until……):

(I=inactive, H = hyperactive, Si = Lying on side, O = other orientation issue/vestibular)

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **A** | | | | | | |
| **B** | | | | | | |
| **C** | | | | | | |
| **D** | | | | | | |

**HEARING TEST PARADIGM:**

START TIME: ……

40 Hz (540 milliseconds, 2.3 Vpp, -15dB)…… (tick)

50 Hz (440 milliseconds, 1.6 Vpp, -15dB)…… (tick)

100 Hz (440 milliseconds, 2.1 Vpp, -25dB)…… (tick)

150 Hz (440 milliseconds, 2.7 Vpp, -35dB)……... (tick)

200 Hz (440 milliseconds, 2.2 Vpp, -30dB)…… (tick)

300 Hz (440 milliseconds, 3.8 Vpp, -40dB)…… (tick)

400 Hz (440 milliseconds, 4.3 Vpp, -41.25dB)…….. (tick)

500 Hz (440 milliseconds, 4.5 Vpp, -30dB)…… (tick)

END TIME: ……..

SAVED AS FOLDER:

……

**RHEOTAXIS AFTER TREATMENT:** (tick)

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **A** | | | | | | |
| **C** | | | | | | |
| **B** | | | | | | |
| **D** | | | | | | |

**MTC AFTER TREATMENT:**

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **A** | | | | | | |
| **B** | | | | | | |
| **C** | | | | | | |
| **D** | | | | | | |

**PLATE 3 PARADIGM:**

Plate 3 placed into startle arena at: ……..

Baseline recording taken for ……. at: …… (30fps)

Room Temp at start: ……. Room Temp at end: ……

**OBSERVATIONS** (over 10 minutes from……..until…….……):

(I=inactive, H = hyperactive, Si = Lying on side, O = other orientation issue/vestibular)

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **A** | | | | | | |
| **B** | | | | | | |
| **C** | | | | | | |
| **D** | | | | | | |

**HEARING TEST PARADIGM:**

START TIME: ……

40 Hz (540 milliseconds, 2.3 Vpp, -15dB)…… (tick)

50 Hz (440 milliseconds, 1.6 Vpp, -15dB)……. (tick)

100 Hz (440 milliseconds, 2.1 Vpp, -25dB)…… (tick)

150 Hz (440 milliseconds, 2.7 Vpp, -35dB)……... (tick)

200 Hz (440 milliseconds, 2.2 Vpp, -30dB)…… (tick)

300 Hz (440 milliseconds, 3.8 Vpp, -40dB)…… (tick)

400 Hz (440 milliseconds, 4.3 Vpp, -41.25dB)…….. (tick)

500 Hz (440 milliseconds, 4.5 Vpp, -30dB)…… (tick)

END TIME: ……..

SAVED AS FOLDER:

……

**RHEOTAXIS AFTER TREATMENT:** (tick)

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **A** | | | | | | |
| **B** | | | | | | |
| **C** | | | | | | |
| **D** | | | | | | |

**MTC AFTER TREATMENT:**

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **A** | | | | | | |
| **B** | | | | | | |
| **C** | | | | | | |
| **D** | | | | | | |

**NOTES/COMMENTS:**

# Appendix 3 Preliminary Data

Towards the end of the research project, the potential effects of cisplatin on ROS induction were investigated, with initial experiments focussing on the effects of cisplatin on the NOX family of enzymes. Some preliminary data are described below.

**mRNA expression of the NOX family of enzymes in the zebrafish**

Methods

*RNA extraction*

Anaesthetised larvae (up to 5 dpf) were transferred into clean 1.5 mL microcentrifuge tubes (STARLAB, Milton Keynes, UK). The number of larvae varied depending on age at extraction (between 10 and 20). All fluid was removed from the larvae and they were 'snap' frozen on dry ice (at this stage, samples could be stored at -80ºC). 500 µL of Trizol (Invitrogen; 15596-026) was added to the tube and the embryos were homogenised using a syringe. The sample was left at room temperature for 5 minutes before adding 100 µL of chloroform. Tubes were immediately and vigorously shaken by hand for 15 seconds and then left to stand for a further 3 minutes at room temperature. After the incubation, samples were centrifuged at 12,000 g for 15 minutes at 4ºC. Approximately 300 µL of the aqueous phase was transferred into a clean, labelled 1.5 mL tube and 250 µL isopropanol added before briefly vortexing the mixture. The sample was left to incubate for 10 minutes at room temperature and then centrifuged at 12,000 g for 15 minutes at 4ºC. The supernatant was removed, leaving a pellet of RNA, which was then washed with 1 mL of 70% ethanol/30% DEPC-treated water. Tubes were centrifuged at 4ºC for a further 5 minutes at 7,500 g before removing the ethanol and air drying for 15 minutes. The pellet was resuspended in 20 µL of DEPC-treated water. 1 µL of the RNA preparation was tested on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) using a scan of the absorbance from approximately 200 nm up to 350 nm. The sample was taken as pure if it had an A260/280 ratio of between 1.8 and 2.1. Aliquots of the pure RNA sample were stored at -80ºC for later use.

*cDNA synthesis*

First strand cDNA synthesis was performed from total extracted RNA using the SuperScript™ III Reverse Transcriptase Kit (Invitrogen), according to the manufacturer's instructions. Total extracted RNA was primed using oligo(dT) and RNA removed at the end using RNase H. The synthesised cDNA was stored at -20ºC and later used for RT-PCR.

*Primer design*

Genes implicated in cisplatin-induced ototoxicity in cell-line or mammalian studies were identified by literature searching. A BLAST search was performed comparing the nucleotide sequence of the candidate gene against the zebrafish genome using the NCBI browser (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The top matches from the BLASTn were viewed in the zebrafish Zv9 genome database on Ensembl (http://www.ensembl.org/Danio_rerio/Info/Index). Primers to potential zebrafish homologues were designed using the NCBI browser (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?) and checked for secondary structures and primer dimers using the Sigma design tool (http://www.sigmaaldrich.com/configurator/servlet/DesignTool?prod_type=STANDARD). These primers were used to amplify gene fragments from zebrafish cDNA by RT-PCR. For details of the primers, see Table A3.1.

**Table A3.1:** Primer design.

| Gene | Number of primer pairs designed | Fragment length (bp) and number of primer pair | Primers to Sequence (5' to 3') |
|---|---|---|---|
| *cyba* | 2 | 473<br>Pair 1 | Forward: TGTGGGCCAACGAGCAAGCTT<br>Reverse: TTTACGGCGCAGTTCAGGGGG |
| | | 457<br>Pair 2 | Forward: TCCCCCTGAACTGCGCCGTA<br>Reverse:<br>ACAAGTCATCGAGAGGACACAAAAGG |
| *Duox1* | 3 | 563<br>Pair 2 | Forward: GGGACACGAGCACGGGCAAA<br>Reverse: ACCAGGGGGCGCAAGAGTCA |
| | | 279<br>Pair 3 | Forward: GTTGGCTTTGGTGTAACTGTA<br>Reverse: GCCCAGGCTGTGAGAG |
| *nox5-like* | 3 | 565<br>Pair 1 | Forward: TGGCATCGGCTGGGTCAACG<br>Reverse: CGCTCTGCTTTGGTGCCGTCT |
| | | 438<br>Pair 3 | Forward: AGTCGTTTTTCGCGGAGCGGTT<br>Reverse: ATGCGCGTGTCAGATAGCGGG |
| *eef1a1l1* | 1 | 543 | Forward: TCTGTTGAGATGCACCACGA<br>Reverse: TGGAACGGTGTGATTGAGG |

*PCR*

DNA sequences were amplified from cDNA using specific primers designed to anneal to the DNA template on either side of the sequence of interest. A standard PCR reaction mixture was used:

**Single reaction:**

2 µL 10x PCR buffer (100 mM Tris-Cl (pH 8.8 at 25 ºC), 500 mM KCl, 1% Triton X-100; Yorkshire Bioscience Ltd., UK)

0.6 µL 50 mM $MgCl_2$ (Yorkshire Bioscience Ltd., UK)

15.25 µL $dH_2O$

0.2 µL 25 mM deoxynucleotide triphosphate (dNTP) mix (Promega)

0.75 µL 10 µM forward primer (Sigma, UK)

0.75 µL 10 µM reverse primer (Sigma, UK)

0.2 µL 5 u/µl DNA Polymerase

0.25 µL cDNA)

For routine PCR procedures, the DNA Polymerase used was YB Taq (Yorkshire Bioscience Ltd., UK). Negative controls used were: forward primer only, reverse primer only and no template cDNA. Primers designed to the constitutively expressed zebrafish elongation factor 1-alpha (*eef1a1l1*) gene were used as a positive control.

The PCR reaction was as detailed in Table A3.2. A thermal gradient of 48 - 64ºC was used to optimise the annealing step. The reaction was performed using an MJ Research PTC-200 Thermo Cycler (Bio-Rad Laboratories Ltd., UK). PCR products were visualised by agarose gel electrophoresis.

**Table A3.2:** PCR reaction

| PCR step | Temperature (ºC) | Time (minutes) | Cycles | Function |
|---|---|---|---|---|
| Denature | 94 | 3 | 1 | Denature the template cDNA (removal of secondary structure) by breaking hydrogen bonds |
| Denature | 94 | 30 | 33 | Melts the template and newly-synthesised cDNA (after cycle 1) by breaking hydrogen bonds |
| Anneal | 48-64 | 45 | 33 | Primers anneal to single stranded template |
| Elongate | 72 | 1 | 33 | Allows Taq polymerase to elongate from the primers, making a copy. |
| Final elongation | 72 | 3 | 1 | Allows remaining single stranded DNA to become fully extended |

*Agarose gel electrophoresis*

Gel electrophoresis was used to check the size and/or quantities of restriction digested DNA and PCR products and to confirm RNA transcription. Agarose (Web Scientific Ltd.) was dissolved into 1X Tris-borate EDTA buffer (TBE) at a concentration of 1-3% by heating. 1µL of 10 mg/mL ethidium bromide solution (BDH Laboratory Supplies, Poole, UK) was added to 30 mL of the molten agarose solution in order to visualise the product of interest. Gels were set in

trays containing combs to form the DNA loading wells. Once set, the agarose gels were immersed in an electrophoresis tank filled with 1X TBE buffer. DNA loading buffer (Fermentas, UK) was added to the product of interest at a final concentration of 1X and products were loaded into the wells. Products were compared to a reference DNA ladder run on the same gel (GeneRuler 100 bp or 1 kb 0.5 mg/mL DNA ladder; Fermentas, UK). In the majority of cases, electrophoresis was performed at 90V for 45 minutes using a Powerpac 300 power supply (Bio-Rad Laboratories Ltd., UK). Samples run on the gel were subsequently visualised under ultraviolet light using a gel doc system.

Results

Primers were designed to the zebrafish mRNA sequence for zfCYBA, zfDUOX-1 and zfNOX-5-like. PCR products could be seen for each of these genes of interest. The PCR product for *zfCYBA* could be observed using cDNA that was a mix of fish aged 3-5 dpf. The PCR products for *zfNOX-5*-like and *zfDUOX-1* could be seen at 26 hpf but were more difficult to detect between 3 and 5 dpf (data not shown). The PCR products were gel extracted and cloned before sequencing. Sequencing showed the expected products in all cases for each gene of interest.

**Protective effects of NOX family inhibitors**

Methods

*MTC testing*

MTC testing of DPI was carried out as in Section 2.4.3 of Chapter 2.

*Compound exposures*

For protection studies, 2 hour co-treatments of cisplatin ($IC_{50}$/450µM/900µM) and DPI at varying concentrations up to half of the MTC concentration were performed (0-62.5 µM, MTC was 125 µM). After compound exposure, larvae were rinsed 3 times with E3 and left to recover for an additional 60 minutes prior to further processing (DASPEI assay, as described in Chapter 2 Section 2.7).

Results

DPI (diphenylene iodonium) is a known inhibitor of the NADPH oxidase family of enzymes. The effect of DPI co-treatment on cisplatin induced hair cell damage was investigated in the posterior lateral line at 5 dpf. Co-incubation of DPI and cisplatin for 2 hours partially protected hair cells of the neuromast against cisplatin–induced decreases in DASPEI staining (Figure A3.1 A). This protective effect was statistically significant at both 450 µM and 900 µM of cisplatin treatment (Figures A3.1 B and C). The data suggest that the NOX/DUOX pathway may be important in the induction of hair cell death in the zebrafish, supporting what is already known from studies in other species.

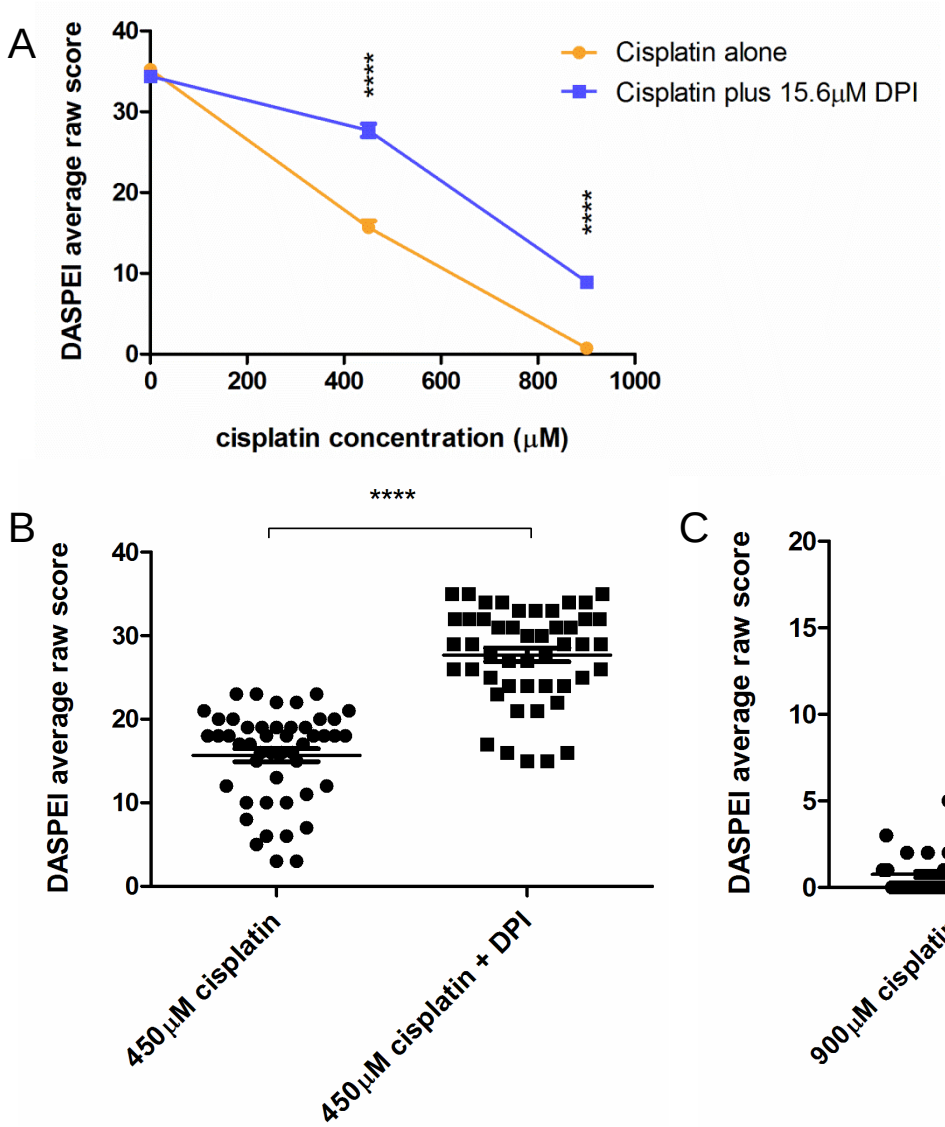
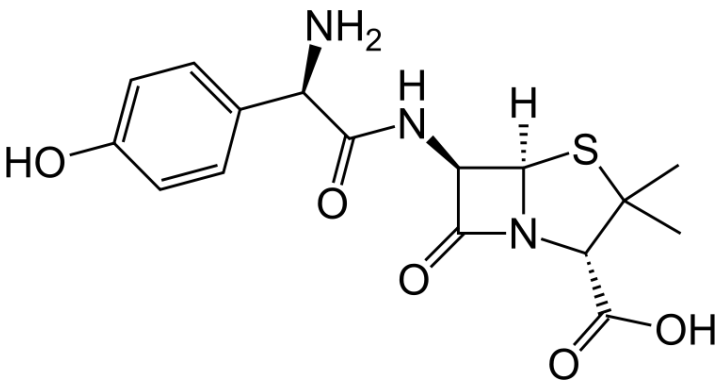


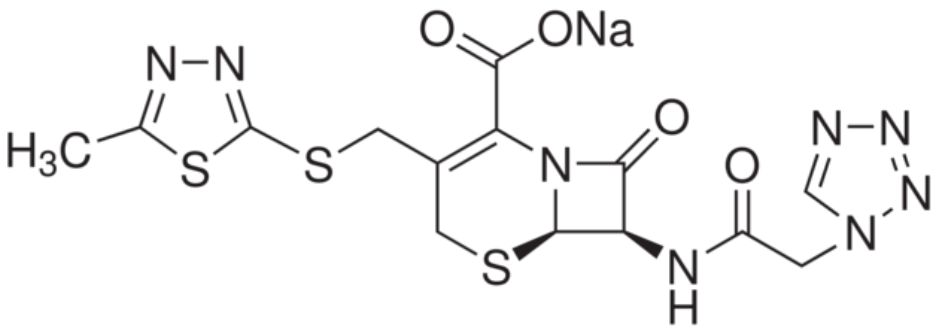

**Figure A3.1: The protective effect of the pan-NOX family inhibitor DPI on larvae treated with cisplatin.** Co-incubation of DPI and cisplatin for 2 hours partially protects against cisplatin–induced decreases in DASPEI staining in the posterior lateral line (A). The protective effect of DPI can be seen at both 450μM (B) and 900μM (C) of cisplatin treatment. For each treatment group *n* is a minimum of 36 larvae, pooled from three separate trials.

# Appendix 4

**A**




**B**




**C**

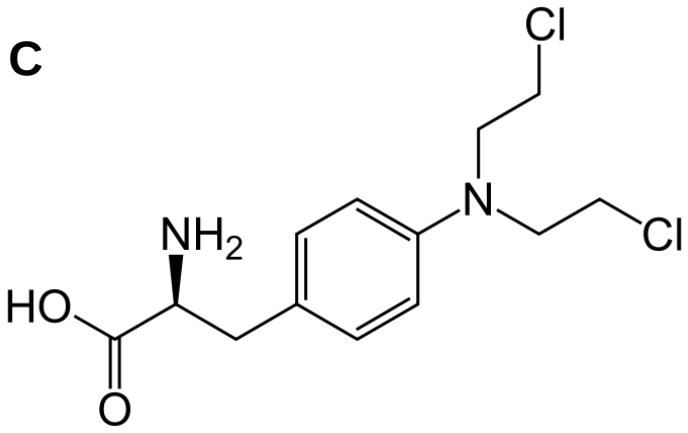
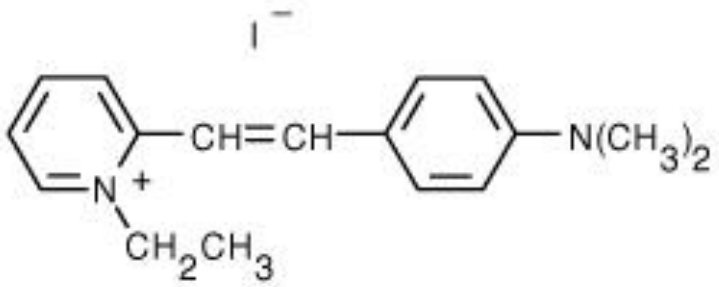


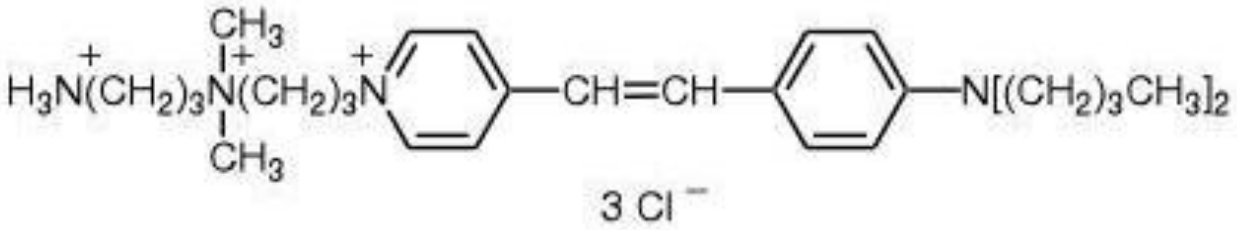

**Figure A4.1: The chemical structures of the negative control compounds.** (A) Amoxicillin, (B) Cefazolin sodium salt and (C) Melphalan hydrochloride.

# Appendix 5

A




B




C

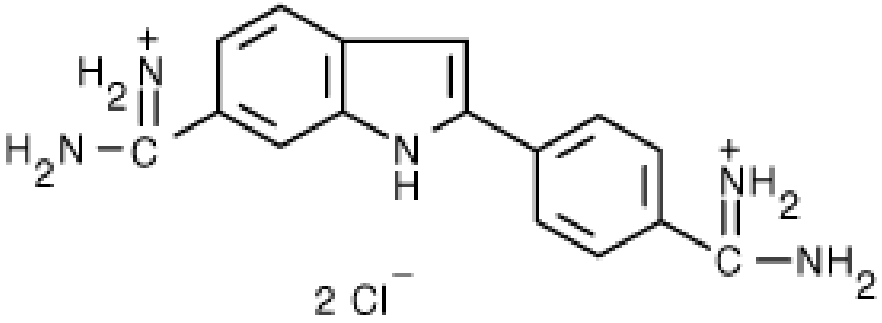



**Figure A5.1: The chemical structures of the vital dyes used.** (A) DASPEI, (B) FM1-43FX, (C) DAPI.